University of Otago

RESEARCH REPORT

Myofilament Mechanisms Underlie Changes of Diabetic Cardiac Impairment

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Abstract

The myofilament protein, cardiac troponin I (cTnI), is a critical regulatory protein in the contraction and relaxation of cardiac muscle, linking calcium to bind to the thin filament. It has become apparent that myofilament proteins and intracellular calcium availability have an effect on the dynamic modulation on contractile function. The phosphorylation of cTnI at specific serine and threonine residue sites is an important physiological mechanism for altered myofilament functional properties and may play a significant role in the contractile dysfunction observed in diabetes. It is possible that exercise might reverse myofilament dysfunction in the diabetic heart by altering cTnI phosphorylation levels. However, whether exercise alters cardiac myofilament proteins in diabetes is unexplored.

This study aimed to investigate the role of cTnI in the diabetic heart and determine the effects diabetes and exercise might have on calcium sensitivity and phosphorylation of the myofilament protein, cTnI. I hypothesised that myofilament phosphorylation and calcium sensitivity are dysregulated in diabetes, which could be mitigated by exercise training.

Left ventricular tissue –from the Zucker diabetic fatty rat, a model of type 2 diabetes, was used to measure total cTnI expression and levels of phosphorylation within cTnI. Measurements were taken from four groups: non-diabetic sedentary, diabetic sedentary, non-diabetic trained, and diabetic trained. We measured myofilament calcium sensitivity via single-cell contractility experiments and quantified the level of phosphorylation of cTnI via Western blot experiments. Rats were randomized to either eight weeks of treadmill running or a sedentary intervention. This combination of experiments allowed us to determine whether the effects of exercise could reverse expected changes in cTnI phosphorylation measured in diabetic tissue.

Western blot experiments from 12-week old tissue illustrated levels of cTnI phosphorylation at serine 150 were decreased in diabetic animals compared to non-diabetic littermates ($P < 0.05$, $n = 5$). In diabetic samples, phosphorylation at serine 23/24 was increased ($P < 0.05$, $n = 10$), however phosphorylation at threonine 143 ($P = 0.21$, $n = 5$) showed an increasing trend but did not reach significance. Single-cell contractility experiments revealed a leftward shift in the calcium-binding curve of diabetic trained cardiomyocytes, indicating an increase in calcium sensitivity compared to non-diabetic trained cardiomyocytes; however, the results were not significant. Immunoblots from 20-week old tissue revealed that cTnI phosphorylation at serine 23/24 ($P < 0.05$, $n = 6$) and threonine 143 ($P < 0.05$, $n = 6$) were significantly decreased in trained diabetics compared to trained non-diabetics, corresponding to an increase in calcium sensitivity.

Our results demonstrate that diabetes dysregulates phosphorylation of the myofilament protein, cTnI. Whereby, this relationship was altered in trained diabetic rats, showing exercise reversed the levels of phosphorylation observed at 12-weeks of age. This thesis has provided a deeper insight into the critical role that cTnI phosphorylation (at numerous amino acid residues) plays in the altering of single-cell functionality in the diabetic heart and its ability to be reversed, at least in part, by exercise.

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List of Abbreviations

%	Per cent
#	Number
<	Less than
>	Greater than
±	Plus/minus
-	Negative
™	Trademark
°C	Degrees celsius
ß	Beta
µ	Micro
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
AMPK	Adenosine monophosphate-activated protein kinase
AMP	Adenosine monophosphate
APS	Ammonium persulfate
β-AR	Beta-adrenergic receptor
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CO	Cardiac output
C-terminus	Carboxyl-terminus (-COOH)
cTnI	Cardiac troponin I
Da	Dalton
DM	Diabetic
ECL	Enhanced chemiluminescence
g	Gram
H_2O	Water
h	Hours
HIIT	High-intensity interval training
HRP	Horseradish peroxidase
k	Kilo
L	Litre
LDA	Length-dependent activation
METs	Metabolic equivalent
M	Mol
min	Minutes
m	Milli
mm	Millimetre
mM	Millimolar
mV	Millivolts
n	Nano
n	Number

nDM	Non-diabetic
N-terminus	Amino-terminus ($-NH_2$)
O_2	Oxygen
O-GlcNAc	O-linked N-acetylglucosamine
PA	Physical activity
PAGE	Polyacrylamide gel electrophoresis
pCa	-log10 of the calcium concentration
pCa_{50}	Calcium required for half-maximal activation
pH	-log[H+]
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PTMs	Post-translational modifications
RIPA buffer	Radio immune precipitation assay buffer
RPM	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
SED	Sedentary
SEM	Standard error of the mean
Ser	Serine
STZ	Streptozotocin
SV	Stroke volume
TBST	Tris-buffered saline with Tween 20
TEMED	Tetramethylethylenediamine
Thr	Threonine
TnC	Troponin C
TnI	Troponin I
TnT	Troponin T
V	Volt
VO_2max	Maximum oxygen consumption
WT	Wild type
ZDF	Zucker diabetic fatty animals

1 Introduction

1.1 Diabetes Mellitus

1.1.1 Characterisation of Diabetes Mellitus

Once a disease prominent amongst only affluent adult populations, diabetes mellitus has spread beyond its traditional confines, now encompassing the poor and, due to increasingly high rates of child obesity, adolescent populations. Diabetes is a chronic, metabolic disease characterised by hyperglycemia due to the result of defective insulin secretion or insulin sensitivity (Mellitus, 2005). There are several types of diabetes, type 1 diabetes, gestational diabetes and, the focus of this thesis, type 2 diabetes. Type 2 diabetes describes defects in insulin secretion caused by dysfunctional pancreatic ß-cells and insulin resistance in target organs (Chatterjee *et al.*, 2017).

1.1.2 Prevalence of Diabetes Mellitus

The prevalence of diabetes globally is continuously rising, showing a four-fold increase in diagnosis numbers from 1980 to 2014 (Zhou *et al.*, 2016). According to the International Diabetes Federation, approximately 463 million adults are living with diabetes, and this number is expected to exceed 700 million individuals, by 2045 (Williams *et al.*, 2019). In New Zealand, it was estimated that in 2017/2018, 253, 000 individuals had diabetes (Ministry of Health, 2019). In particular, type 2 diabetes affects approximately 90% of diabetes cases (Williams *et al.*, 2019). The reality whereby such a large proportion of the population has type 2 diabetes should arguably come as no surprise, especially considering the increasingly sedentary lifestyle, inadequate nutrition and rapid urbanisation (Hu, 2011).

1.1.3 Heart Disease and Mortality of Diabetes Mellitus

Chronic hyperglycemia can have detrimental effects throughout several organ systems of the body, manifesting in complications such as neuropathy, retinopathy, nephropathy and most important to this review, cardiovascular disease. In 1972 it was first identified that diabetic patients had increased risk of cardiomyopathies independent of other risk factors, including hypertension, high serum cholesterol levels, age, and weight (Rubler *et al.*, 1972). Likewise, confirmed in the Framingham study, diabetic patients appeared to be two to five times greater at risk of developing cardiomyopathies than their counterparts (Kannel & McGee, 1979).

Currently, literature tends to agree that a significant cause of mortality and morbidity in patients with type 2 diabetes is cardiovascular disease (Gu *et al.*, 1998). This mortality and morbidity are commonly attributed to the reduction in contractility of the left ventricle as a consequence of type 2 diabetes (Jweied *et al.*, 2005; Baldi *et al.*, 2006). At present, the diabetic heart phenotype is defined as the existence of abnormal myocardial structure and function in the absence of other commodities, such as coronary artery disease and hypertension (Boudina & Abel, 2007). Moreover, data indicates that the prevalence of type 2 diabetes is increasing rapidly (Williams *et al.*, 2019); thus, illustrating the need to further focus our efforts on unravelling the underlying mechanisms.

1.2 Cardiac Structure and Calcium Signalling

1.2.1 Basics of Heart Function

The heart is an intermittent muscular pump, divided into four chambers; left and right atrium, and left and right ventricle. The right ventricle pumps blood to the lungs, while the left ventricle simultaneously pumps blood to the rest of the body.

With every heartbeat, cells in the body are provided with sufficient levels of oxygen as a consequence of the ventricles ejecting blood into circulation pathways of the body (Bers, 2002). Cardiac output, a product of stroke volume (the amount of blood ejected per heartbeat) and heart rate, relies on the systolic (contracting) phase of the cardiac cycle, and the diastolic (relaxation) phase of the cardiac cycle. The diastolic phase becomes more significant due to its ability to increase end-diastolic volume during stress, for example, which occurs with exercise (van der Velden, 2011). One key mechanism for controlling contraction and relaxation of the heart is regulating the availability or amount of intracellular calcium (Ca^{2+}) present.

1.2.2 Excitation-Contraction Coupling

Cardiac excitation-contraction coupling is the process of electrical excitation of the myocyte to contraction of the heart (Figure 1.1) (Bers, 2002). As a second messenger, Ca^{2+} is essential for cardiac electrical stimulation, resulting in a contraction by activating the myofilaments. Evidence suggests that Ca^{2+} mishandling is a common trigger for both contractile dysfunction and cardiovascular diseases (Antoons *et al.*, 2002).

Upon the depolarisation of the cardiomyocytes, Ca^{2+} enters the cell through L-type Ca^{2+} channels, triggering the opening of the ryanodine receptor which releases Ca^{2+} from the sarcoplasmic reticulum. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increases, allowing Ca^{2+} to bind to the myofilament protein troponin C, initiating myofilament contraction through conformational changes in myofilament structure (Figure 1.1). Relaxation of the cardiomyocytes occurs when the $[Ca^{2+}]_i$ decreases drastically, allowing Ca^{2+} to dissociate from troponin C via removal of Ca^{2+}. This occurs primarily through the reuptake of Ca^{2+} into the sarcoplasmic reticulum (Figure 1.1).

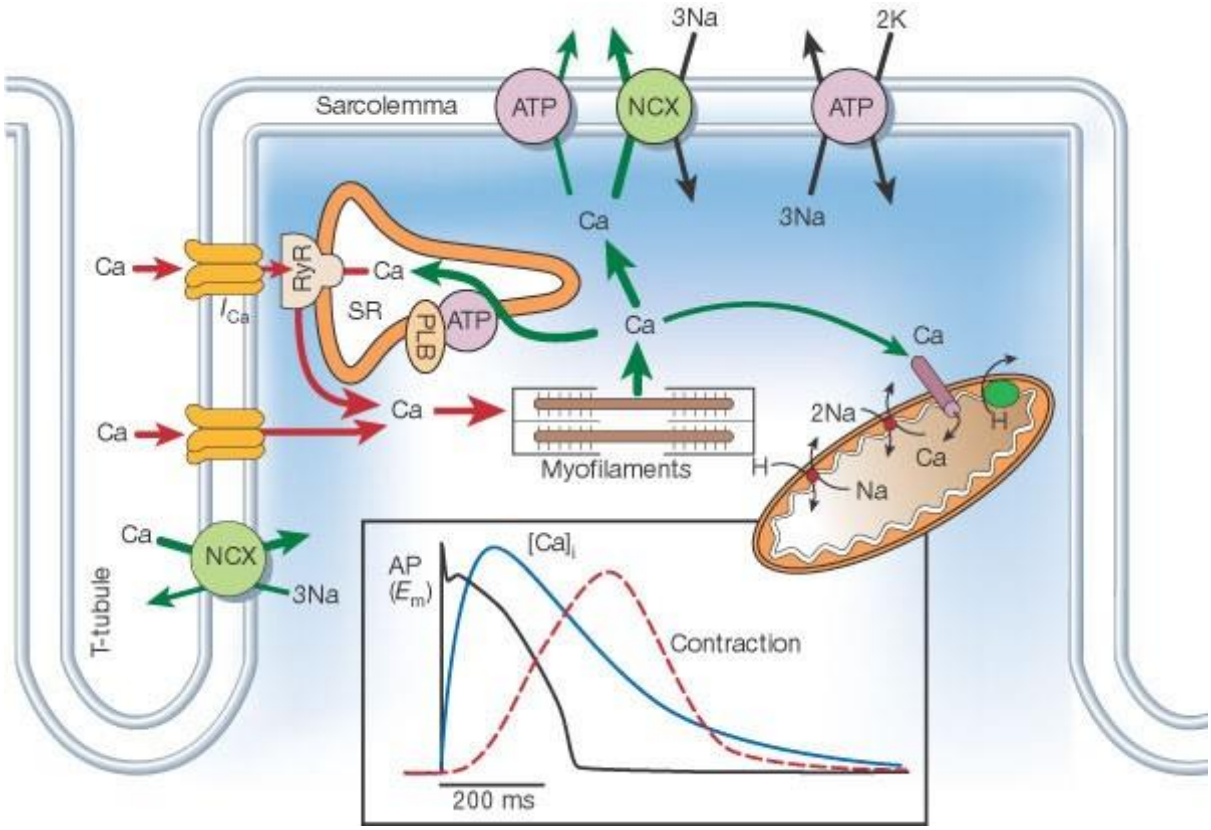

Figure 1.1. Schematic of Ca^{2+} homeostasis and excitation-contraction coupling. *An action potential is fired as myocytes are excitable which triggers the depolarization of the sarcolemma along its t-tubules. This triggers inward Ca^{2+} influx through LTCCs, leading to the opening of ryanodine receptor (RYR2) channels by a Ca^{2+}-induced-Ca^{2+}-release process, resulting in Ca^{2+} release from the sarcoplasmic reticulum (SR). This Ca^{2+} release binds to the actin filament, facilitating the formation of cross-bridges and muscle contraction. Relaxation occurs when Ca^{2+} is re-up taken into the SR and is extruded from the cell by the sarcolemma Na^+ and Ca^{2+} exchanger (NCX). (Adapted from Bers, 2002).*

1.2.3 Cardiac Muscle

The heart is made up of cardiac muscle tissue, which consists primarily of cardiac myocytes. Myocardial tissue is striated, as a result of the regular arrangement of contractile proteins, thin actin and thick myosin filaments arranged in functional units called sarcomeres. These contractile proteins in the cardiomyocytes interact due to an ‘unlocking’ mechanism, providing them with the ability to generate force and contraction. The principal regulator of muscle contraction is one of the components of the thin filament called the troponin complex; made up of troponin C (TnC), troponin T (TnT), and troponin I (TnI) (Figure 2). The inhibitory unit of the troponin complex is the thin filament protein TnI, which acts to inhibit actomyosin interactions at diastolic levels of intracellular Ca^{2+}. The elevation of Ca^{2+} in

systole relieves the inhibitory influence of cardiac troponin I (cTnI) through binding to TnC, thus stimulating an actomyosin cross-bridge contraction.

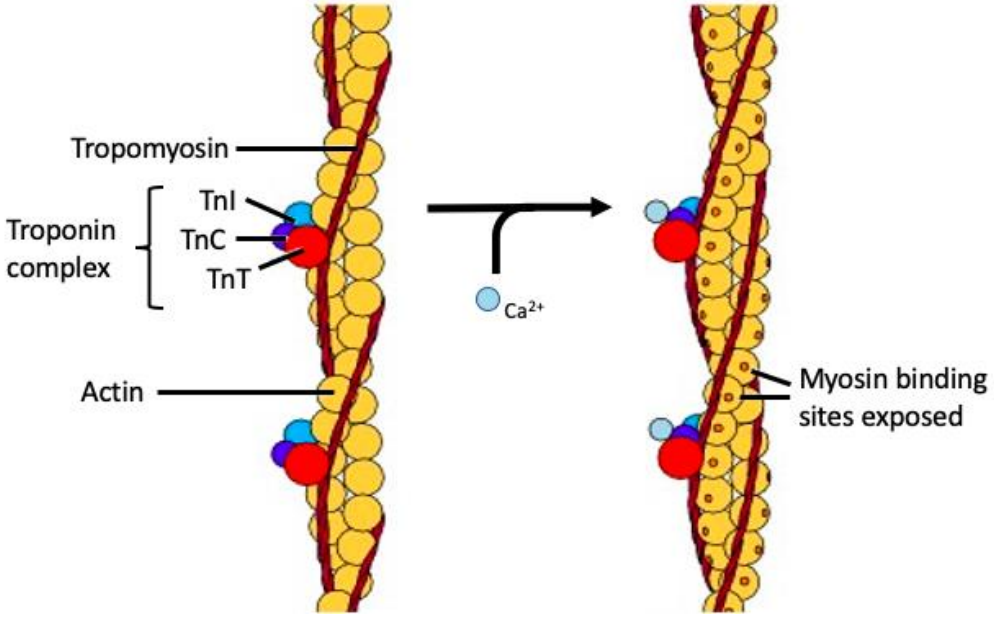

Figure 1.2. Structural view of the cardiomyocyte. *This figure illustrates how cardiomyocytes contain thin filaments consisting of actin monomers and thick filaments consisting of myosin and myosin heads. Tropomyosin wraps around the actin filaments and is associated with a complex of three troponins; troponin I (TnI), Troponin C (TnC), and troponin T (TnT). The binding of Ca^{2+} to TnC allows for the tropomyosin-troponin complex to shift off the actin filament. Myosin can now bind to the thin filament, allowing the contraction to proceed. (Adapted from Cooper & Hausman, 2000).*

Tropomyosin wraps around the actin filaments, blocking the myosin-binding sites and is associated with the troponin complex (Figure 1.2). The binding of Ca^{2+} to TnC allows for the tropomyosin-troponin complex to shift off the actin filament allowing myosin to bind to the thin filament, resulting in the contraction (Figure 1.3). Once Ca^{2+} dissociates from TnC tropomyosin wraps itself around the actin again blocking the myosin-binding sites, and the sarcomere relaxes. Lastly, besides Ca^{2+}, the availability of ATP for ATP-hydrolysis is also a regulatory mechanism of contraction (Li *et al.*, 2003).

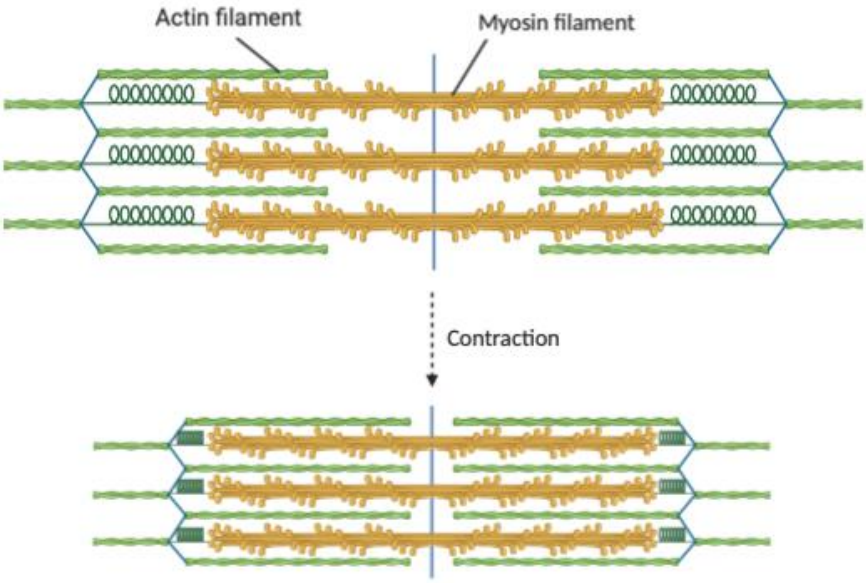

Figure 1.3. Schematic drawing of a myocardial contraction. *This diagram shows the actin filaments sliding past the myosin filaments towards the middle of the sarcomere, forming a cross-bridge cycle. This action results in the muscle contracting during systole. (Original figure Gold, 2020).*

1.2.4 The Effects of Diabetes on the Contractility of the Diabetic Heart

Heart patients with diabetes frequently have an impaired left ventricular function relative to non-diabetic patients (Group, 2013). Consequently, a normal healthy lifestyle is compromised due to the increased stiffness and diastolic dysfunction of the left ventricle, followed by systolic impairment, resulting in the inability of type 2 diabetics to increase systolic function during exercise or other stressors (Baldi *et al.*, 2016). Consistent results from research using diabetic (db/db) mice as a model of type 2 diabetes show that when measuring contractile performances of perfused Langendoff hearts and Ca^{2+} transients using isolated cardiomyocytes, these resulted in a reduction in both the peak rate of force production and total force production (Belke *et al.*, 2004; Stolen *et al.*, 2009). These investigations support the idea that in diabetic cardiomyopathy, intrinsic alterations of cardiac myocytes are

impairing the heart's ability to relax which likely represents the initial stage of diabetic cardiomyopathies.

In pursuit of understanding the underlying pathogenesis of cardiovascular diseases, research (Diffee *et al.*, 2001) has revealed that Ca^{2+} sensitivity is critical for the force generation of the left ventricle. Comparisons of left ventricular cells from heart patients with and without type 2 diabetes suggest that diabetic cardiomyocytes have reduced Ca^{2+} sensitivity (Jweied *et al.*, 2005). It was thought that to increase the cardiac contractility, a drug that increased $[Ca^{2+}]_i$ through eliciting a sequence of events involving cAMP was necessary. New insights show that these drugs generally fail to increase contractility in diabetic patients, suggesting that the reduced Ca^{2+} availability is not the underlying problem (Malik *et al.*, 2011).

At present, there is a lack of work testing diabetes-specific changes in the cardiomyocyte contractile apparatus even though diabetic myofilament function might be a novel therapeutic target (reviewed by Baldi *et al.*, 2016).

1.3 Post-translational Modifications

1.3.1 Post-translational Modifications Affect Heart Function

Myocardial contractility represents the innate ability of the heart muscle to eject a stroke volume at a given afterload and preload. At the cellular level, the determinants of contractility are the generation of tension and shortening capabilities of the sarcomere through cross-bridge kinetics as controlled by Ca^{2+} activation, the rate of cross-bridge turnover, and the relative Ca^{2+} response in the sarcomere. Post-translational modifications are changes to a protein following translation whereby the addition of functional groups or proteins, proteolytic cleavage of regulatory groups, or the degradation of proteins can be the explanation of protein regulation of acute changes in disease susceptibility (McLarty *et al.*,

2013). In the heart, post-translational modifications can alter myofilament function, specifically the ability of Ca^{2+} to bind to TnC. One common type of post-translational modification is phosphorylation, a reversible reaction that occurs as a consequence of protein kinases and the addition of a phosphoryl group (Figure 1.4) (Rodney, 2006). Altered phosphorylation at myofilament proteins affects Ca^{2+} sensitivity in other aetiologies of heart failure (Marston & de Tombe, 2008).

Cellular Ca^{2+} cycling and myofilament properties are under strict control via phosphatases and kinases within the hearts muscle cells (Hamdani *et al.*, 2008). Thus, these proteins dephosphorylate and phosphorylate target proteins in the myofilament, a pair of processes that play a role in the regulation of contraction and relaxation (Neumann *et al.*, 1997). Phosphorylation of troponin is suggested to change Ca^{2+} affinity in other forms of heart failure (Takeishi *et al.*, 1998; Dong *et al.*, 2012). In particular, troponin is of interest to this review due to several works showing the functional significance of phosphorylation of troponin in models of heart failure.

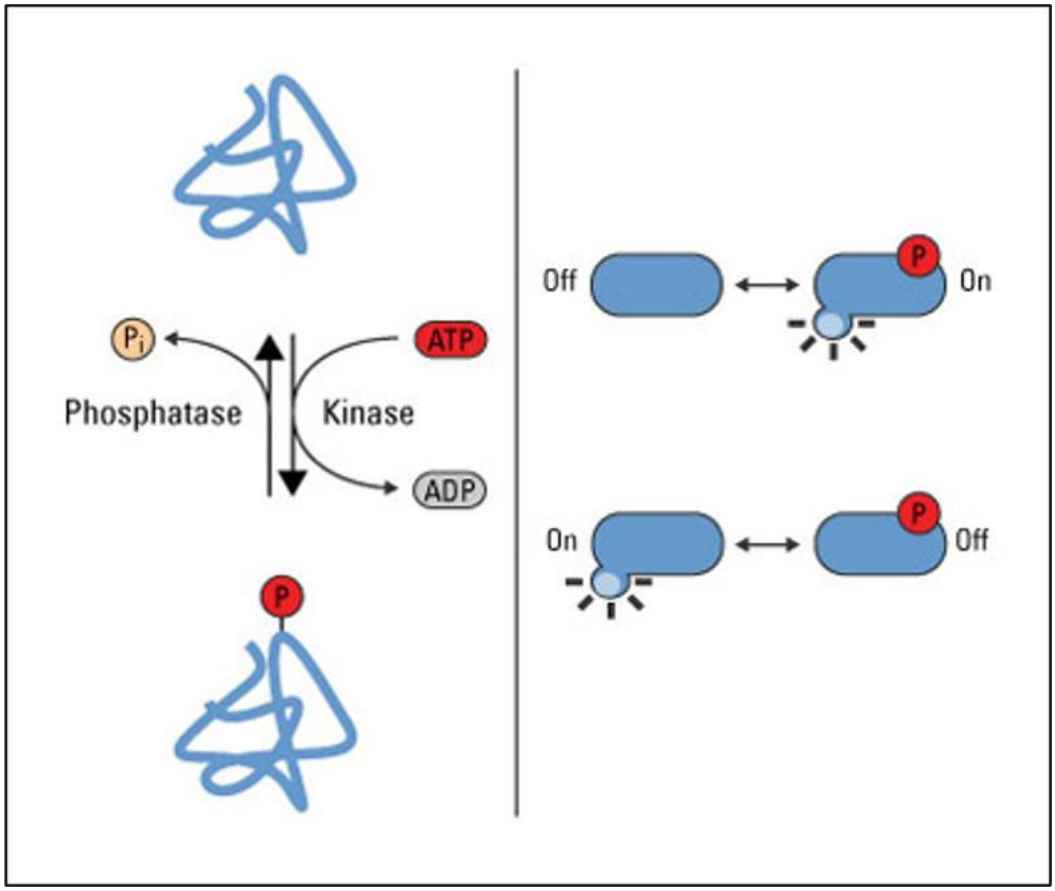

Figure 1.4. Phosphorylation mechanism. The left panel shows the reversibility of phosphorylation, a post-translational modification, regulating protein function. Protein kinases mediate phosphorylation at specific side chains, and phosphatases reverse protein phosphorylation by hydrolysing the phosphate group. The right panel shows that the addition of a phosphate group causes a conformational change in proteins that either activates or deactivates protein function. (Adapted from *ThermoFisher Sci*).

1.4 The Myofilament Underlies Changes to Contractility

1.4.1 Cardiac Troponin I and the Myofilament

At present, cTnI is a crucial regulator of cardiac muscle contraction and relaxation. This is commonly attributed to the Ca^{2+} sensitivity of the myofilament being governed by the expression of cTnI of the cardiac myofilament (Layland *et al.*, 2004). Conversely, variations in contractility of the myocardium can be a result of changes to the Ca^{2+} transient size or duration. Despite this, evidence from mice treated with gene-targeted ablation of cTnI demonstrated a reduction in myofilament Ca^{2+} sensitivity, diastolic dysfunction and the

development of heart failure (Huang *et al.*, 1999). This work suggests the importance of cTnI in health and disease.

1.4.2 Phosphorylation of Cardiac Troponin I

cTnI function is altered by PTMs such as phosphorylation and O-GlcNAcylation. The phosphorylation of cTnI at specific serine (Ser) and threonine (Thr) residues underpins the mechanisms through which myofilament properties are altered (Figure 1.5B). These modifications in cTnI phosphorylation status could reflect changes in the balance between kinases and phosphatases, with altered expression being reported in failing hearts (Bodor *et al.*, 1997a; van der Velden *et al.*, 2003). Predominantly, β-adrenergic stimulation promotes cAMP-dependent protein kinase A (PKA)-mediated phosphorylation of cTnI. This stimulates an increase in the circulatory system, often altering the properties of the cardiac myofilament proteins, including myofilament Ca^{2+} sensitivity, to meet the physiological demands of stressors, through positive inotropic and lusitropic effects (Layland *et al.*, 2005).

Myofilament Ca^{2+} sensitivity (often expressed as pCa_{50}) is a functional measurement that indicates the necessary Ca^{2+} concentration within the cytosol required to generate 50% maximal tension, which models physiological Ca^{2+} levels during systole. For example, PKA-mediated phosphorylation of the myofilament decreases pCa_{50} and increases maximal tension (Zhang *et al.*, 1995; Pi *et al.*, 2003), essential factors of cardiac contractility.

A common finding in the literature investigating Ca^{2+} sensitivity in human cardiomyocytes is increased Ca^{2+} sensitivity is associated with diseased samples compared to control sample obtained from organ donors (Bodor *et al.*, 1997a; van der Velden *et al.*, 2003). The increase is pCa_{50} in diseased hearts has been found to be associated with low cTnI

phosphorylation. Previous studies have linked amino acid residues within cTnI with increased Ca^{2+} sensitivity in the failing heart, including PKA sites Ser23/24 (Messer *et al.*, 2007), PKC sites Thr143 and Ser43/45 (Wijnker *et al.*, 2014), and AMPK sites Ser150 (Sancho Solis *et al.*, 2011) (Figure 1.5A). It has been strongly suggested in the literature that these phosphorylation sites either contribute to or attempt to compensate for, the altered myofilament functioning in failing cardiomyocytes (van der Velden *et al.*, 2003).

There is a growing body of literature that recognises phosphorylation of myofilament proteins in heart failure (Marston & de Tombe, 2008) and its role in modulating myofilament Ca^{2+} sensitivity (Layland *et al.*, 2005). However, few studies have investigated the effects phosphorylation may have on the diabetic heart. In a streptozotocin (STZ) mouse model of diabetes, Jweied and colleagues found that cTnI and cTnT phosphorylation was augmented but could not correlate these findings with Ca^{2+} sensitivity (Jweied *et al.*, 2005). Although this study was completed in a mouse model and not human, the results indicate that phosphorylation might contribute to the functional changes occurring in the diabetic heart. In the STZ rat model of diabetes, augmented pCa_{50} corresponded with no change in the amount of phosphorylation at key myofilament functional proteins like myosin-binding protein C and myosin regulatory light chain 2 (Falcao-Pires *et al.*, 2011). However, in the same rats, results indicated there was significant compensation for metabolic changes, further contributing to the decreased contractility measured. To date, there is still work needed to assess both phosphorylation status and function in skinned cardiomyocytes in the Zucker diabetic fatty (ZDF) rat model of diabetes.

As previously mentioned, kinases and phosphatases are responsible for phosphorylation activity, and the mechanisms that underpin this may lead to

hyperphosphorylation or hypophosphorylation in the diabetic heart. For example, β-adrenergic receptor (β-AR) protein expression is reduced in the ZDF heart (Haley *et al.*, 2015), suggesting a reduction in PKA mediated phosphorylation in the ZDF heart. Additionally, extensive research has shown that PKC phosphorylation is increased in diabetic hearts (Malhotra & Sanghi, 1997; Ahmad *et al.*, 2005), and that PKC phosphorylates cTnI and cTnT (Noland *et al.*, 1995). Collectively, there appears to be a plethora of evidence demonstrating phosphorylation of myofilament proteins, including cTnI, contribute to myofilament dysfunction, and together affect organ-level dysfunction of contractility in the diabetic heart.

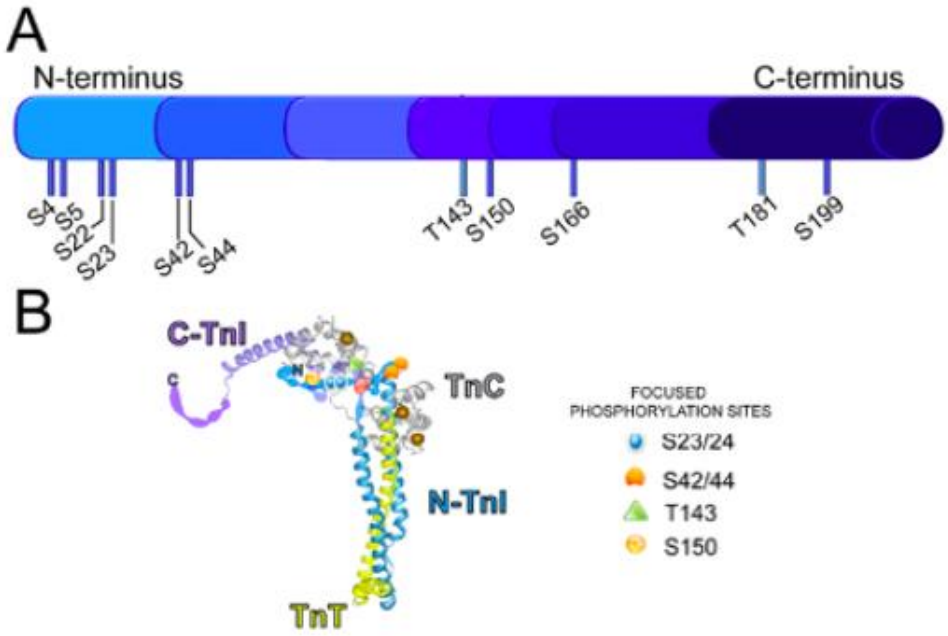

Figure 1.5. Structural view of the cTnI. *(A) Position of cTnI phosphorylated Ser (S), and Thr (T) residues. (B) Structural model of cTnI, troponin T (TnT), and Troponin C (TnC) showing the location of phosphorylation sites. (Adapted from Takeda et al., 2003).*

Based on previous research, the phosphorylation of cTnI at Ser23/24, Thr143 and Ser150 are ideal candidates for the investigation of altered myofilament properties in diabetic cardiomyocytes to identify potential novel therapeutic targets.

1.4.3 Troponin and the Diabetic Heart

A common characteristic of type 2 diabetes is the progressive development of a cardiovascular disease, commonly manifesting as an impairment in diastolic performance (Baldi *et al.*, 2006). The intrinsic properties of the myofilaments appear to be altered (Malhotra & Sanghi, 1997), in particular, the myofilament Ca^{2+} sensitivity. Although Ca^{2+} sensitivity research has been implicated in non-diabetic patients with heart failure (Bilchick *et al.*, 2007), research is just beginning to be thoroughly done in the diabetic heart. In heart failure, initial studies isolating cells from end-stage failing hearts showed alterations in Ca^{2+} sensitivity (van der Velden *et al.*, 2003). These changes in myofilament Ca^{2+} sensitivity have been linked to post-translational modifications including defects in the β-adrenergic signalling pathway due to a reduction in phosphorylation of PKA protein targets (Bodor *et al.*, 1997b), previously described in the above section. Alongside reduced PKA-mediated protein phosphorylation, numerous myofilament protein modifications have been reported, which may be the underlying cause of impaired diastolic function of the heart (Wijnker *et al.*, 2011; Wende, 2016). The post-translational modifications, such as phosphorylation, and O-GlcNAcylation, show alterations within the diabetic heart.

Although studies identifying reduced Ca^{2+} sensitivity in type 2 diabetics is limited, research has been carried out in human biopsies and type 1 diabetic rat models. In the diabetic heart, the most common finding is reduced Ca^{2+} sensitivity (Jweied *et al.*, 2005) tested using a methodology involving skinned cells attached to a force transducer measuring force generated in different concentrations of Ca^{2+}. Jweied et al. (2005) used human myocardial biopsies, of which patients were diabetic and non-diabetic. Their study assessed for myofilament function using the skinning methodology and their results showed a decreased Ca^{2+} sensitivity, that is,

a depressed myofilament function, associated with the diabetic patients. Their results demonstrated that cTnI and TnT might be constitutively phosphorylated in the diabetic heart; thus, myofilament function and Ca^{2+} sensitivity might be expected to be depressed.

Despite these results, when considering the ZDF rat model of diabetes, pCa_{50} was not different between non-diabetic and diabetic ZDF rats at 16 weeks of age (van den Brom *et al.*, 2009). Regardless of the lack of a pCa_{50} difference between groups, diabetic ZDF rats had a reduced rate of cross-bridge cycling, which might explain, in part, the myofilament functional changes leading to these rats impaired diastolic and systolic functioning. Indicating that the diastolic and systolic dysfunction present early in the ZDF rat, may not be due to altered Ca^{2+} sensitivity, but instead, a result of other functional parameters associated with the maximal force generated or the rate of cross-bridge cycling. Nevertheless, the conflicting data within the diabetic heart and Ca^{2+} sensitivity emphasize the need for more studies to better understand the cause or the adaptations of the disease.

1.5 Exercise Recommendations and Lifestyle Interventions

1.5.1 Physical Activity

It is widely agreed that regular physical activity is a fundamental component of a healthy lifestyle (Pate *et al.*, 1995). Physical activity can be defined as "any bodily movement produced by skeletal muscles that result in energy expenditure" (Caspersen *et al.*, 1985). Over 25 years ago, the American College of Sports Medicine issued physical activity guidelines to clarify the regulatory and intensity to improve health, lower susceptibility to diseases and decrease rates of early mortality. They quantified the intensity of physical activity as metabolic equivalents or METs and recommended moderate-intensity activity per day, which equates to an activity performed with a 3-6 METs intensity. Regardless of the scientific evidence linking physical activity to health benefits, (Kannel & McGee, 1979), as well as the

apparent public acknowledgement surrounding the importance of physical activity, millions of individuals remain sedentary. Several clinical trials have confirmed the effects of regular exercise on patients with chronic heart failure, showing it can improve the prognosis of these patients including improvements in cardiac structure and function (Cassidy *et al.*, 2016), reduced cardiovascular risk (Morris & Crawford, 1958), increase longevity (Lee *et al.*, 2017), and increased quality of life (Antonicelli *et al.*, 2016).

1.5.2 Exercise as an Intervention

It is well-established that exercise training status and exercise capacity predict the risk of cardiovascular disease and mortality (Myers *et al.*, 2002). Multiple studies, including the Framingham Study, the Harvard Alumni study, and the Lipid Research Clinics Trial all demonstrated that exercise testing provides the ability to determine mortality rates, as there is a graded inverse relationship between total physical activity and mortality. It has been proven through research that poor physical fitness is modifiable, and overtime increased exercise capacity has demonstrated improved prognostics of cardiovascular diseases and diabetes (Pate *et al.*, 1995). Observations of treadmill running have shown that for every 1-MET increase in performance, there is a resulting 12% improvement in survival in patients with or without cardiovascular disease (Myers *et al.*, 2002). Other studies such as The Dallas Bed Rest and Training Study are contributing to this growing body of evidence, demonstrating that regular exercise training increases aerobic capacity (VO_2max), stroke volume and cardiac output in deconditioned individuals (Saltin *et al.*, 1968). Despite this, the specific cellular alterations are behind those organ-level function changes are still largely unknown.

1.5.3 Diabetes and Exercise

Individuals with type 2 diabetes often present with lower VO_2max when compared to non-diabetic individuals (Baldi *et al.*, 2003). However, literature comparing the oxygen extraction in diabetics and non-diabetics is both limited and with contradictory results (Baldi *et al.*, 2006; Mac *et al.*, 2011). Nevertheless, the literature agrees that a reduction in exercising cardiac output is characteristic of individuals with type 2 diabetes (Baldi *et al.*, 2003; Baldi *et al.*, 2010; O'Connor *et al.*, 2015). Similar to that of a healthy individual, a diabetic's resting cardiac output remains identical to that of a non-diabetic individual. Patients with type 2 diabetes do, however, typically achieve this similar cardiac output with a faster heart rate and lower left ventricular stroke volume than a non-diabetic individual (Baldi *et al.*, 2003; Pinto *et al.*, 2014). During exercise, a diabetic patients heart rate reserve is reduced (Mac *et al.*, 2011) and their left ventricular stroke volume either increased slightly or not at all (Baldi *et al.*, 2006). These factors influence the oxygen-carrying capacity, which ultimately affects the VO_2 max of an individual with type 2 diabetes or the ability to reach higher workloads.

Exercise has been well established as an intervention for cardiovascular disease, primarily focusing on the lifestyle adaptations with physical activity as the target in mind (Lear *et al.*, 2017). Diffee et al. (2001) trained rats to undergo a progressive treadmill running protocol for 11 weeks and performed experiments on chemically skinned myocytes to determine the force and Ca^{2+} sensitivity of the myofilaments. The results indicated that the heart responds to exercise training in ways that increased Ca^{2+} sensitivity.

Subsequently, regular endurance exercise training elicits positive adaptations of cardiac function that enhance the cardiovascular system by improving the maximal and submaximal performance of the left ventricle (Diffee & Nagle, 2003), due to the improvement

of the intrinsic contractility of the myocardium (Diffee *et al.*, 2001). Thus, the effects of exercise on the diabetic heart should be tested, as it could prove to improve the contractility of the diabetic heart.

1.6 Aims and Hypotheses

Firstly, this thesis aimed to determine the phosphorylation status of the myofilament protein cTnI in ZDF diabetic hearts. Further, we aimed to associate changes in cTnI phosphorylation with pCa_{50}. We hypothesized that in ZDF rats' diabetic hearts, we would observe an increase in cTnI phosphorylation at Ser23/24, and Thr143, and a decrease at Ser150. We also hypothesized that diabetes would induce a reduction in pCa_{50} in the ZDF rats hearts.

Secondly, we aimed to determine the effects of exercise training on my phospho-state of cTnI in non-diabetic and diabetic hearts. We hypothesized that exercise training would increase pCa_{50} in both non-diabetic and diabetic hearts. We also hypothesized that an increase in pCa_{50} would correspond with a decrease in Ser23/24, Thr143 and increase in Ser150 phosphorylation.

2 Methods

2.1 Rat Model of Diabetes

2.1.1 Zucker Diabetic Fatty rat (ZDF)

The ZDF rats used in this study are a standard animal model of type-II diabetes (Pang *et al.*, 2018). The ZDF rat has a gene coding mutation on the leptin receptor (fa/fa) which can lead to obesity and hyperglycaemia when on a high-fat chow diet (Phillips *et al.*, 1996; Pick *et al.*, 1998). This genetic modification is considered a model of early-onset diabetes as they exhibit hyperglycaemia, hyperinsulinemia, and hypertriglyceridemia conditions at about 12 weeks of age (Ikeda *et al.*, 1981). Throughout this thesis, homozygous ZDF rats will be referred to as diabetic (DM), and the lean rats will be referred to as non-diabetic (nDM). The non-diabetic littermates consist of homozygous dominant (+/+) or heterozygous (fa/+) genotypes and are normoglycaemic throughout their lifespan. In this study, both 12 and 20-week-old rats were used to determine cTnI phosphorylation at the initial stages of diabetes and cardiac myofilament sensitivity after eight weeks of uncontrolled diabetes.

2.1.2 Animal Handling & Tissue Collection

Single-cell contractility experiments, and exercising protocols described below were all performed by *Ms* Angela Greenman (A PhD student in the Department of Medicine, University of Otago). All procedures and animal handling were conducted with the approval of the University of Otago's Animal Ethics Committee and per the New Zealand Animal Welfare Act (1999). All rats were housed in a controlled environment (stable temperature of 21 ± 1ºC and standard 12-hour light and dark cycle) at the animal holding facility, University of Otago, Dunedin. ZDF rats were fed a high-fat Purina 5008 diet (LabDiet, St Louis, MO USA) ad libitum and given water ad libitum.

Animals were anaesthetised with a lethal dose of sodium pentobarbital (60 mg/kg) through intraperitoneal injection. Upon anaesthesia, the final body weight was measured. The chest cavity was opened, and the heart was excised promptly and put into a cold (4ºC) relaxing buffer (100 mM KCL, 1.75 mM EGTA, 10 mM Imidazole, 4 mM ATP, 5 mM $MgCl_2$, pH 7.0 with KOH). The heart was weighed proceeding the removal of excess tissue and then dissected into its four chambers and septum. Left ventricle dissection was completed in less than six minutes upon opening the chest. The left ventricle was weighed and snap-frozen in liquid nitrogen prior to other tissues. All tissues were stored at -80 ºC in preparation for later use.

2.2 Treadmill Running Intervention

2.2.1 Randomisation and Familiarisation

The ZDF rat develops diabetes at ~12 weeks of age but does not develop indices of heart failure until week 20. Therefore, we randomly assigned 12-week old rats (diabetic and non-diabetic littermates) to either an exercise or sedentary intervention. Each pair had two days of acclimatising to the new environment before an initial echocardiography. The rats randomised to the exercise intervention experienced a familiarity period with the rodent treadmill (Ugo Basile, Figure 2.1) which included three sessions of 10-, 12.5- and 15-minutes spread over three different days. To ensure familiarisation, a speed of 5 m/min was used to train the rat on how the belt moves. They were also familiarised to a 5% grade which was used throughout the intervention.

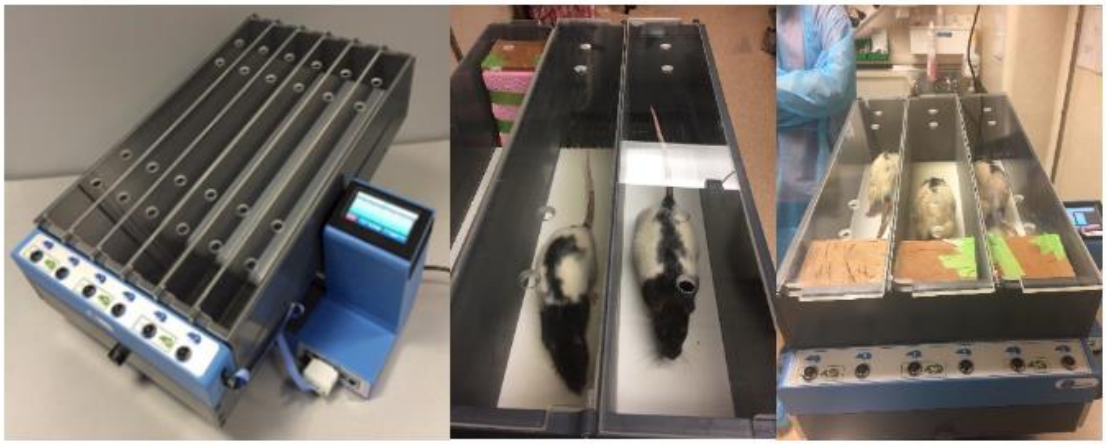

Figure 2.1. Image of the rodent treadmill *(left). ZDF rats running without wooden blocks (middle) and, with wooden blocks and sponges (right) to motivate the rats and protect their tails from abrasions, respectively. (Greenman, 2020, Unpublished).*

2.2.2 Exercise Training

The 8-week treadmill running protocol progressively increased duration and speed to accommodate adaptation to the exercise training. Running duration began at 10 minutes per day but increased to a maximum of 40 minutes per day by the fourth training day. Initially, the speed was set to 10m/min and progressed to 18 m/min by training day 29. The grade was set at 5% throughout the intervention.

2.3 Western Blotting (Immunoblotting)

Many researchers have utilised the western blot technique to identify and detect protein expression. The benefit of this approach is that the different proteins become separated in a gel, based on molecular weight (Shapiro *et al.*, 1967). For this project, the proteins were separated using sodium dodecyl (SDS) and the polyacrylamide gel electrophoresis (PAGE) technique. The expression levels of phosphorylated cTnI residues (Ser23/24, Ser150, and Thr143) normalized to total cTnI were investigated from ZDF rats from both diabetic and non-diabetic as well as sedentary and exercise interventions. ß-actin was used to ensure equal

loading. To visualise the proteins, gels containing the proteins need to be transferred from the SDS-PAGE gel to a nitrocellulose membrane, as detailed in section 2.3.6. Once transferred onto the membrane, proteins of interest could be visualised via the addition of specific antibodies.

2.3.1 Sample Homogenisation

Prior to left ventricular tissue being placed in a radioimmunoprecipitation assay (RIPA) buffer for sample homogenisation, 1% SDS RIPA buffer solution was prepared (composition: 50 mM Tris-HCl, 150 mM NaCl, 1% Triton, at pH 7.5), supplemented with inhibitors (200 µL 25X cOmplete, 500 µL 20X PhosphSTOP, 55 µL 100 mM PMSF, 400 µL 10% SDS) to prevent protein degradation. A higher concentration of SDS was required to adequately isolate myofilament proteins of interest (i.e., troponin). Left ventricle tissue was cut into smaller pieces (~50 mg) and added to glass tubes, ensuring the tissue was fully covered by RIPA buffer plus inhibitors solution (~500 µL). The tissue was mechanically homogenised on ice by the IKA Ultra Turrax T8 homogenizer at speed 8 for 3 x 30 seconds, or until sample appeared completely homogenised. The homogenate was transferred to centrifuge tubes and incubated on ice prior to centrifugation (15000 RPM, 15 min, 4ºC). The supernatant containing protein was carefully transferred to new Eppendorf tubes, appropriately labelled. Protein concentration was determined using Gen5 Take3 protein quantification assay from BioTek. Duplicates of ~3 µL of homogenate samples were loaded onto the microplate spectrophotometer. Quantification of the protein concentration from the sample is estimated by the amount of light absorbed at 280 nm wavelength (Whitaker & Granum, 1980). Preceding, protein samples were snap-frozen in liquid nitrogen and stored in the -80ºC freezer.

2.3.2 Sodium Sulfate Poly-Acrylamide Gel Electrophoresis

SDS PAGE allows for electrophoretic protein separation. SDS is a detergent that denatures proteins and polypeptide chains and donates negative charges to give all proteins an equal charge (Rath *et al.,* 2009). Moreover, SDS can convert proteins into linear structures of different sizes, allowing for the migration of proteins, through the gel, dependent on molecular weight (Figure 2.2) as well as facilitating binding to amino acids and giving a negative charge to the primary structure. The negative charge is then used with the PAGE technique, where the proteins run through the polyacrylamide gel away from the top negative cathode towards the bottom positive anode. The migration of the smaller and larger proteins can be differentiated through applying an electrical field. Polyacrylamide forms a matrix suitable for the separation of proteins of a specific size. The pore sizes in the matrix are determined by the percentage of acrylamide in the gels; thus, protein migration is dependent on an electrical field and pore size. A protein ladder, with known molecular weights, runs parallel with other protein samples and allows for detection of proteins via molecular weight.

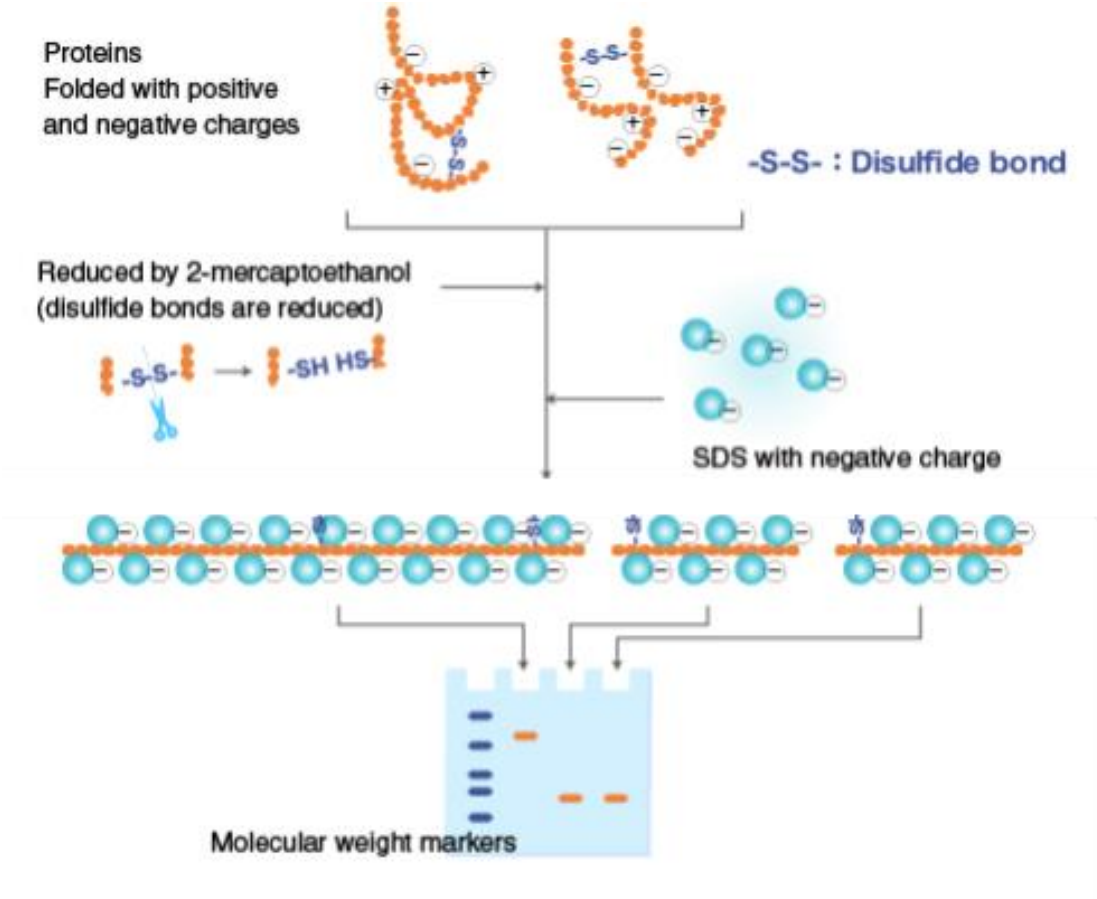

Figure 2.2. Schematic representation of protein denaturation by SDS. *Proteins are in a folded structure with positive and negative charges. In the presence of SDS and a reducing agent that cleaves disulphide binds critical for protein folding, proteins unfold into linear chains with negative charges. (The principle and method of polyacrylamide gel electrophoresis (SDS-PAGE), n.d.).*

2.3.3 Gel Casting

SDS-PAGE electrophoresis was carried out using the Mini-Protean III system (BioRad, UK). Here, 15% polyacrylamide resolving gels and 4% polyacrylamide stacking gels were prepared according to Table 2.1, using glass plates (BioRad, UK) with 1.5 mm spacers (BioRad, UK). To ensure the gel would mould correctly, the cast was filled with water and left to stand for 15 minutes to check for no leakage.

Table 2.1: Components for 15% resolving gel and 4% stacking gel. *The volumes used for each component of a 1 x 1.5mm gel for SDS-PAGE experiments.*

Components	**Resolving gel 15% (µL)**	**Stacking gel 4% (µL)**
Milli-Q H_2O	2832	2516
1.5M Resolving Buffer (375 mM Tris, pH 8.8)	2000	N/A
0.5M Stacking Buffer (125 mM Tris, pH 6.8)	N/A	1000
40% Acrylamide	3000	400
10% SDS	80	40
10% Ammonium persulfate (APS)	80	40
Tetramethylethylenediamine (TEMED)	8	4

Initially, a 15% resolving gel was cast, with an additional water layer added to remove air bubbles and prevent evaporation. The gel was left to polymerise for 1 hour at room temperature (RT). Once set, the overlying water layer was removed, and a 4% stacking gel was loaded on top of the resolving gel, and a 15 well comb was inserted to form the loading wells (Figure 2.3). The stacking gel was left to set for 1 hour. The plates containing the gels were inserted into the running tank, immersed in 1x running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS) and the combs were removed.

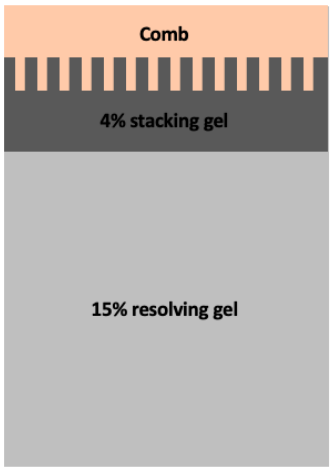

Figure 2.3. Structural view of the SDS-PAGE gel*. (Gold, 2020, original figure).*

2.3.4 Sample Preparation

A protein quantity of 20 µg/lane was chosen for this study. A total of 10 µL was loaded per lane, which consisted of 20 µg of protein per sample, a final concentration of 1X sample buffer (31.25 mM Tris pH 6.8, 10% glycerol, 2% SDS, 500 mM DTT, 0.01 mM Bromophenol blue) and water adjusted to make up the final volume. Following, the samples were heated to 90ºC for 5 minutes to ensure protein denaturation and then centrifuged (13500 RPM for 0.5 minutes) to collect the sample at the bottom of the Eppendorf tube. Samples were kept on ice before loading.

2.3.5 Protein Separation

Precision protein plus dual colour standard (Bio-Rad) also referred to as the molecular weight marker, was loaded into the first well from the left used to monitor the progress of the protein migration. Laemmli-diluted samples were loaded (10 µL/lane). An electrical field (120 V, ~75 minutes) was applied to the gel until the bromophenol blue (leading dye) was near the bottom of the gel.

2.3.6 Wet-Tank Transfer from the gel to a membrane

In this experiment, a BioRad Transfer Unit was used to transfer proteins onto a nitrocellulose membrane. The resolving gel was separated from the stacking gel and placed on top of the membrane, sandwiched between pre-cut filter paper and sponges on either side of the sandwich (Figure 2.4). Prior to the formation of the transfer sandwich, filter papers, sponges and nitrocellulose membranes were soaked in 1x transfer buffer (192 mM Glycine, 25 mM Tris). Between the addition of each component of the transfer sandwich, a roller was run to remove any potential air bubbles that could confound the transfer. Lastly, the transfer buffer was poured into the transfer tank, and the cathode lid was placed on top. The gels were run with an Amersham™ Electrophoresis Power Supply – EPS 301 (GE Healthcare, Buckinghamshire, UK) for 3 hours at 100 V, located in the cold room (4ºC). This moved proteins from the gel to the membrane, where they became attached and immobilised.

Figure 2.4. Schematic view of the membrane transfer 'sandwich' used in a wet-transfer*. The 'sandwich' facilitates the migration of the negatively charged proteins towards the positive anode from the polyacrylamide gel onto the nitrocellulose membrane. (Gold, 2020, original figure).*

2.3.7 Membrane Blocking and Probing with Antibodies

After the transfer was complete, the nitrocellulose membrane was blocked in 5% skimmed milk dissolved Tris-buffered saline (1x TBST: 150 mM NaCl, 50 mM Tris, 0.05% Tween-20, pH 7.6), to prevent non-specific binding of the antibodies.

Hereafter, the membrane was cut horizontally, guided by the protein ladder to liberate the bottom containing cTnI (~28 kDa), and a top portion where β-actin (~42 kDa) was present. Once the membrane was cut, the bottom membrane was probed with its respective primary antibodies (dilutions are shown in Table 2.2) overnight (4ºC, on the rocker). The following morning, the top membrane was probed with its respective primary antibody for 1 hour (RT, on the rocker). After treatment, the membranes underwent 4 x 5-minute washes in TBST; this ensured that unbound antibodies were removed to reduce background hence increase the signal to noise ratio. Primary antibodies recognise and bind to the protein of interest, and the secondary antibody binds to the primary antibody (Figure 2.5).

Figure 2.5. Schematic diagram of probing the membrane with primary and secondary antibodies. *Following blocking the membrane, it is probed with a primary antibody (blue) and then a secondary antibody (red). (Gold, 2020, original figure).*

The bottom membrane was incubated in its respective secondary antibody (dilutions are shown in Table 2.3) for 1 hour (RT, rocker). Secondary antibodies tagged with horseradish peroxidase (HRP) were used to bind to the primary antibody, thus allowing protein detection (Figure 2.5). The top membrane did not require a secondary antibody for the detection of β-actin because the primary antibody is HRP conjugated. Following

administration of the necessary antibodies, the membranes underwent 4 x 5-minute washes, in TBST again.

Table 2.2. *Summary of primary antibodies used, with dilutions factors and incubation periods for related experiments.*

Primary Antibody	**Total cTnI**	**Ser23/24**	**Ser150**	**Thr143**	**β-actin**
Dilution factor	1:1000	1:1000	1:1000	1:1000	1:25,000
Dilution solution	TBST	TBST	TBST	TBST	TBST
Incubation conditions	Overnight, 4ºC	Overnight, 4ºC	Overnight, 4ºC	Overnight, 4ºC	1hr, RT
Manufacturer	Cell signalling technology	Cell signalling technology	Abcam	Abcam	Abcam

Table 2.3. *Corresponding secondary antibodies used, with dilutions factors and incubation periods for related experiments.*

Secondary Antibody	**Total cTnI: Rb-HRP**	**Ser23/24: Rb-HRP**	**Ser150: Rb-HRP**	**Thr143: Rb-HRP**
Dilution factor	1:25,000	1:25,000	1:25,000	1:25,000
Dilution solution	TBST	TBST	TBST	TBST
Incubation conditions	1hr, RT	1hr, RT	1hr, RT	1hr, RT
Manufacturer	Abcam	Abcam	Abcam	Abcam

2.3.8 Protein Detection by Enhanced Chemiluminescence (ECL)

After washing the membrane, it was re-orientated onto cling film on a flat surface. An equal mixture of Reagent A (2 mL) and Reagent B (2 mL) from the Clarity ECL Western Blot

Detection Reagents (Bio-Rad, UK) were mixed and pipetted onto the membrane to fully cover it and left to sit for 5 minutes at RT. The membrane was then placed between two sheets of transparent plastic. HRP catalyses the oxidation of luminol resulting in the light to be released and captured by the SYNGENE PXi machine (Figure 2.6). Varying exposure times were required to optimise band intensity. Captured images were saved and transferred to a computer for band quantification using Genetools (version 4.3.10).

Figure 2.6. Schematic diagram of the chemiluminescence method. *A reaction mixture is added to the membrane. The enzyme (yellow) attached to the antibody catalyses a reaction that emits light, this is detected by a camera. (Gold, 2020, original figure).*

2.3.9 Densitometry

Using image analysis software Genetools (version 4.3.10), a rectangle was placed around the bands of interest, and the software automatically identified the lanes and their respective bands. Densitometry analysis was carried out by normalising the densities of the protein bands of interest to the loading control, β-actin. This was done to account for any potential errors that could have occurred while loading proteins into the SDS-PAGE gels. In experiments analysing specific phosphorylation sites, the density of protein was normalised to the total cTnI protein expression.

2.3.10 Stripping

When probing for specific cTnI phosphorylation sites, a stripping protocol was utilised. Following the imaging of the blot for total cTnI protein expression, it was stripped of that

antibody using a Stripping Buffer (100 mM β-Mercaptoethanol, 2% SDS, 62.5 mM Tris) for 45 minutes at 50ºC. The membrane underwent 4 x 5-minute washes each in TBST, followed by imaging for 10 minutes using the SYNGENE PXi machine to ensure the cTnI antibody had been removed. Once confirmed, the membrane was blocked in 5% skimmed milk dissolved Tris-buffered saline for an additional 1 hour, rinsed with TBST and incubated with the phospho-specific antibody of interest (see Table 2.2) overnight. Sections 2.3.7, 2.3.8, and 2.3.9 were repeated as described above.

2.4 Single-cell Isolations and Calcium Sensitivity Testing

2.4.1 Extraction and Permeabilization of Skinned Cardiomyocytes

Left ventricle samples that had previously been frozen were partially homogenised in ~500 µL relaxing buffer with HALT Protease and Phosphatase Inhibitor Cocktail 100X (Thermo Scientific, 78430). The myocyte suspension was centrifuged at 10, 000 RPM, re-suspended and permeabilised with 1.1% Triton X-100 (Thermo Scientific) for 8 minutes. This resulted in a pellet of skinned myocytes that were washed with relaxing buffer three times. Myocytes were stored on ice and used for up to two days.

2.4.2 Selecting and Gluing the Skinned Cardiomyocytes

A drop of cell suspension was put on a coverslip and placed on top of a glass slide on an inverted phase-contrast microscope (Nikon). The single cardiomyocyte or small bundle of cells (approximately two to five cells) being analysed were then chosen based on good striation pattern and size. It was then aligned with the needle tips of the piezoelectric motor and a force transducer (Aurora Scientific, 405 model). A thin layer of silicone glue (Marineland Aquarium Sealant) was placed on the tips of the force-transducers which were immersed into the drop of cell suspension and attached to the ends of the cardiomyocyte to

create a glue halo around both ends. Once the tips were in position using motorised micromanipulators, they were put down so that they were glued to the extremity of the cardiomyocyte and left there for 10 minutes. After the cardiomyocytes were securely mounted, they were lifted slightly to make sure the force transducers did not stick to the coverslip. The glue was left to cure for a further 30 minutes at a set temperature of 15ºC. Once fixed, the cell could be moved into the well containing the lowest concentration of Ca^{2+} (pCa 9.0) and stretched until the sarcomere length was equivalent to 2.3 µm (Figure 2.7). Sarcomere length, cell length and cell width were visualised and measured using an *IDS* camera and *VSL 900B* software (Aurora Scientific). The experimental set-up was placed on top of a floating table (TMC, USA) to reduce noise.

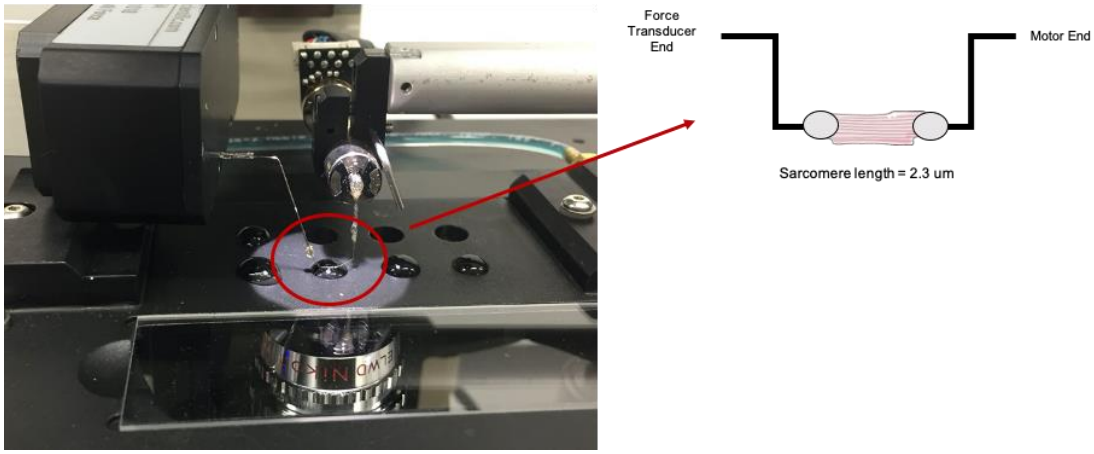

Figure 2.7. Image of the Ca^{2+} concentration bath apparatus. *A schematic diagram of an isolated cardiomyocyte, stretched to 2.3µm when glued to the piezoelectric motor and a force transducer. (Greenman, 2020, Unpublished).*

2.4.3 Recording Force Measurements of Active, Passive and Calcium Sensitivity

The cardiomyocyte was gently moved into baths containing different concentrations of Ca^{2+} to develop myofilament tension. To measure the force at a given pCa, the myocyte was slacked by 20% of its original length (slack test) upon force plateau (Figure 2.8). The change in force from peak length to 80% of its initial length gave a measurement of force as a

given pCa. Initially, force was measured in a Ca^{2+} concentration bath of 4.5 and then randomly selected submaximal Ca^{2+} concentration solutions, with every fourth or fifth activation performed in the 4.5 solution to assess any decline in myocyte performance. Submaximal Ca^{2+} concentration solutions were made by mixing different ratios of Ca^{2+} concentration solutions 9.0 (7.0 mM EGTA, 20.0 mM Imidazole, 5.42 mM $MgCl_2$, 79.16 mM KCL, 16.33 µM $CaCl_2$, 14.5 mM CrP, 4.74 mM ATP, pH 7.0 with KOH) and 4.5 (7.0 mM EGTA, 20.0 mM Imidazole, 5.26 mM $MgCl_2$, 64.0 mM KCL, 7.01 mM $CaCl_2$, 14.5 mM CrP, 4.81 mM ATP, pH 7.0 with KOH), determined from the computer program Fabiato (Fabiato, 1988). The experimenter was blinded to disease-state or intervention assignment of a given left ventricle sample to avoid any bias when choosing to slack the cell. Myocytes were excluded from the data analysis if 80% of their maximal force was not maintained by the end of the protocol.

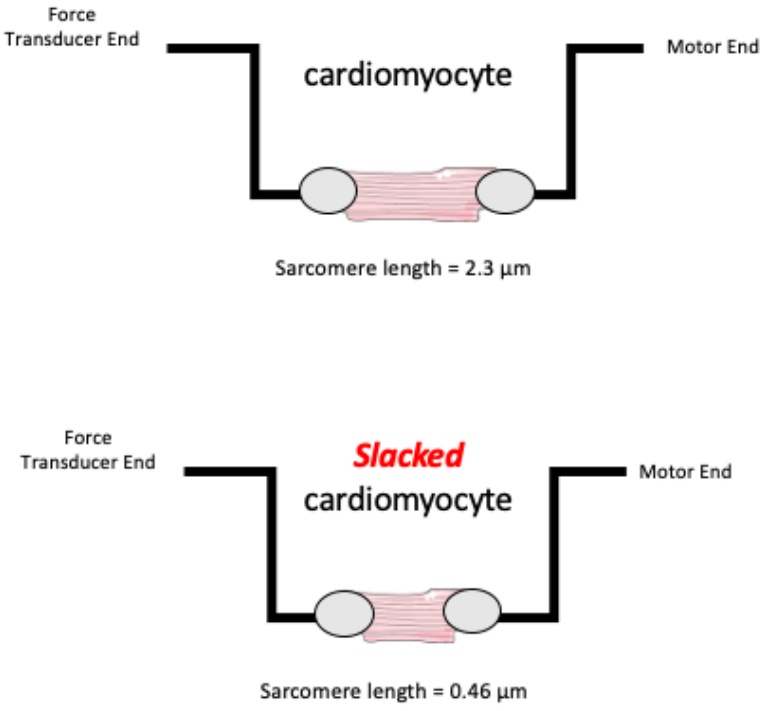

Figure 2.8. Schematic diagram of slacking a cardiomyocyte. *Cardiomyocytes are stretched to a sarcomere length of 2.3µm, and when ready to be slacked, the sarcomere reduces down to 20% of its original size. (Modified from (Greenman, 2020, Unpublished)).*

2.4.4 Calculations of Active Tension

Active tension was calculated by subtracting passive tension at Ca^{2+} concentration 6.0 solution from the total tension measured by the slack test. Tension at the different Ca^{2+} centration solutions was expressed as a fraction of the maximum tension (Ca^{2+} concentration 4.5) obtained under identical conditions for that cell. Data were analysed by least-squares regression using the Hill equation as described by Hofmann et al. (Hofmann *et al.*, 1991),

$$\text{Log}\,[(P_{rel})/(1 - P_{rel})] = n(\log\,[Ca^{2+}] + k)$$

P_{rel} represents the tension expressed as a fraction of maximal tension, *n* is the Hill coefficient, and k is the intercept of the fitted line with the x-axis, which corresponds to the Ca^{2+} concentration at half-maximal tension (pCa_{50}). Tension data were fitted with the following equation using Prism software through using constants derived from the Hill equation.

$$P_{rel} = [Ca^{2+}]^n/(k^n + [Ca^{2+}]^n)$$

2.5 Data Analysis

2.5.1 Statistical Analysis

All statistical analysis was conducted using GraphPad Prism 8 software. A student's t-test (unpaired t-test) was used to compare between diabetic and non-diabetic groups in 12-week old tissue. A two-way Analysis of Variance (ANOVA) was used to compare between non-diabetic sedentary, diabetic sedentary, non-diabetic exercise and, diabetic exercise sample groups with Tukey's post hoc test. Statistical significance was set at $P < 0.05$, and all data were presented as mean ± the standard error of the mean (SEM).

3 Results

3.1 Baseline Analysis of 12-week old nDM and DM Rats

3.1.1 Verification of Total cTnI Expression

A representative blot is presented in 3.1A, showing total cTnI protein bands at 28kDa. Total cTnI was expressed evenly in both diabetic (DM) and non-diabetic (nDM) groups (Figure 3.1A). Figure 3.1B shows a quantitative assessment of total cTnI in tissue from the left ventricle of non-diabetic and diabetic ZDF rats at 12 weeks of age. Total cTnI expression was unchanged in the left ventricle tissue from the diabetic rats compared to the non-diabetic rats (0.81 ± 0.094 vs. 0.82 ± 0.074, nDM vs. DM; Figure 3.1B). Thus, cTnI expression is the same in both diabetic and non-diabetic rats prior to the administration of the exercise program.

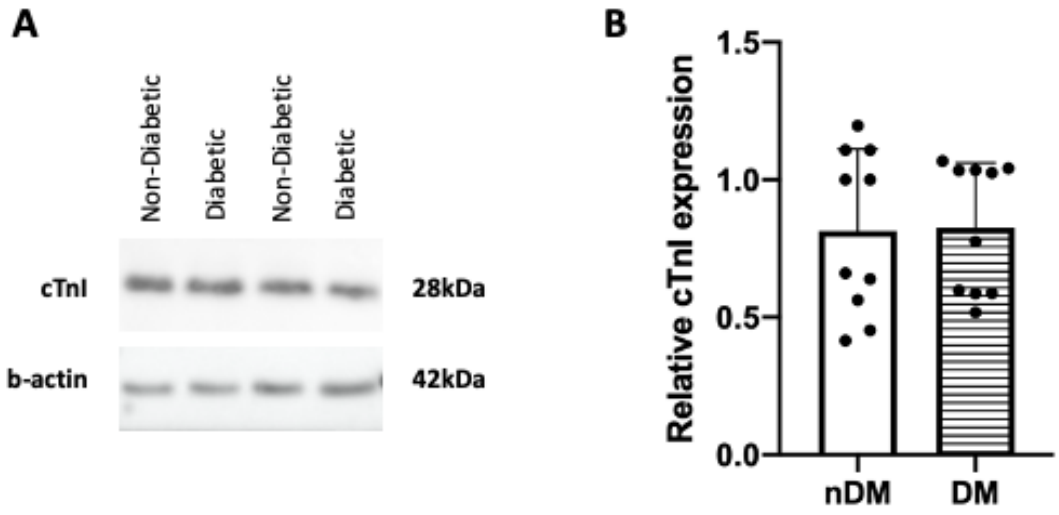

Figure 3.1. Total expression of cardiac troponin I in diabetic and non-diabetic rat hearts at 12 weeks of age*. (A) Representative western blot image of cTnI and β-actin. (B) Individual value plots with a bar graph showing the quantification of cTnI in diabetic (DM) and non-diabetic (nDM) LV tissue of ZDF rats. β-actin was used as an internal control. n = 10 DM samples and 10 nDM samples. Values are represented as mean ± SEM. Statistics calculated with an unpaired, two-tailed, students t-test. All these experiments were performed as duplicates.*

3.1.2 Serine 23/24 Phosphorylation

Figure 3.2A illustrates the level of cTnI phosphorylation at Ser23/24, measured in left ventricle tissue from non-diabetic and diabetic ZDF rats. A quantitative assessment shows that there is a significant increase in the levels of cTnI phosphorylation at Ser23/24 in diabetic cardiomyocytes compared to non-diabetic cardiomyocytes at 12 weeks of age (0.46 ± 0.11 vs. 1.1 ± 0.22, nDM vs. DM, $P < 0.05$; Figure 3.2B). These data support my hypothesis that diabetes leads to an increase in phosphorylation at Ser23/24, potentially affecting the functionality of the myofilament protein, cTnI.

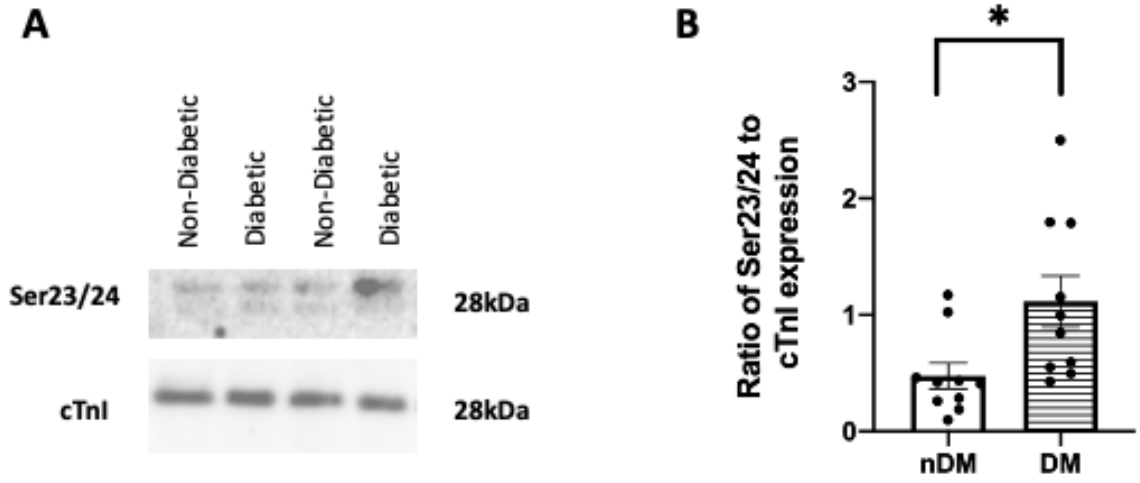

Figure 3.2. Diabetes increases phosphorylation of cTnI at serine 23/24. (*A) Representative western blot images of Ser23/24 phosphorylated and cTnI as a control in DM and nDM left ventricle tissue in 12-week old ZDF rats. (B) Quantitative bar graph showing the levels of phosphorylation at Ser23/24 in diabetic (DM) and non-diabetic (nDM) ZDF rats LV tissue at 12 weeks of age. n = 10 DM samples and 10 nDM samples. Values are represented as mean ± SEM. Statistics calculated with an unpaired, two-tailed, students t-test. All these experiments were performed as duplicates. *P < 0.05 vs. non-diabetic.*

3.1.3 Serine 150 Phosphorylation

Figure 3.3A illustrates the level of cTnI phosphorylation at serine 150, measured in left ventricle tissue from non-diabetic and diabetic ZDF rats. A quantitative assessment shows that diabetic rats had significantly reduced levels of cTnI phosphorylation at Ser150 than that

of non-diabetic rats (3.2 ± 0.87 vs. 0.59 ± 0.15, nDM vs. DM, P < 0.05; Figure 3.3B). These results provide evidence to support my hypothesis that diabetes does result in altered levels of phosphorylation at Ser150.

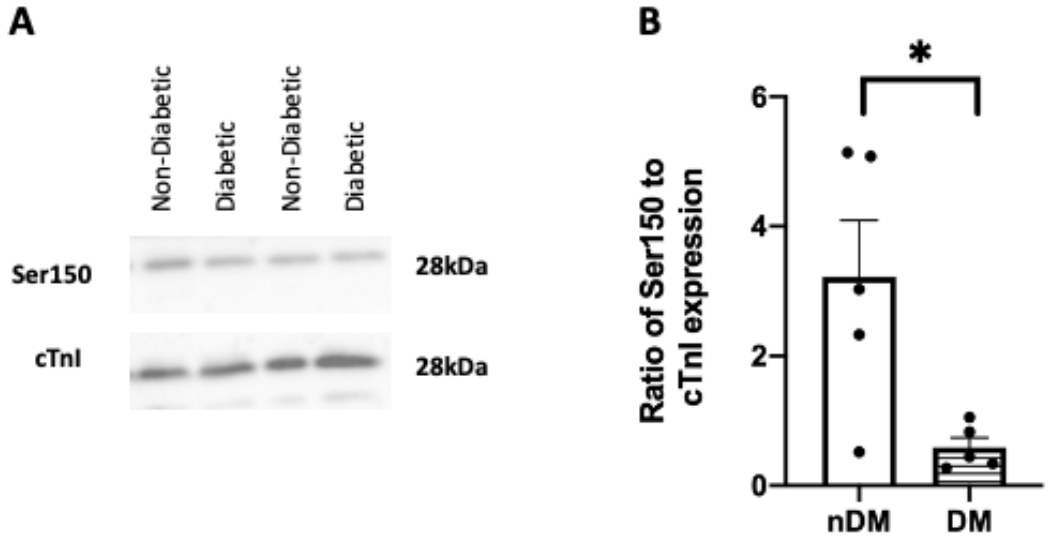

Figure 3.3. Diabetes decreases phosphorylation of cTnI at serine 150. *(A) Representative western blot images of Ser150 phosphorylated and cTnI as a control in diabetic and non-diabetic left ventricle tissue in 12-week old ZDF rats. (B) Quantitative bar graph showing the levels of phosphorylation at Ser23/24 in diabetic (DM) and non-diabetic (nDM) ZDF rats LV tissue at 12 weeks of age. n = 5 DM samples and 5 nDM samples. Values are represented as mean ± SEM. Statistics calculated with an unpaired, two-tailed, students t-test. All these experiments were performed as duplicates. *P < 0.05 vs. non-diabetic.*

3.1.4 Threonine 143 Phosphorylation

Figure 3.4A illustrates the levels of cTnI phosphorylation at Thr143, measured in left ventricle tissue from non-diabetic and diabetic ZDF rats. A quantitative assessment shows no significant differences in the amounts of phosphorylation between non-diabetic and diabetic cardiomyocytes at Thr143 (0.39 ± 0.070 vs. 0.45 ± 0.094, nDM vs. DM, P = 0.61; Figure 3.4B). Therefore, there is not enough evidence to accept my hypothesis that diabetes alters the levels of phosphorylation at Thr143.

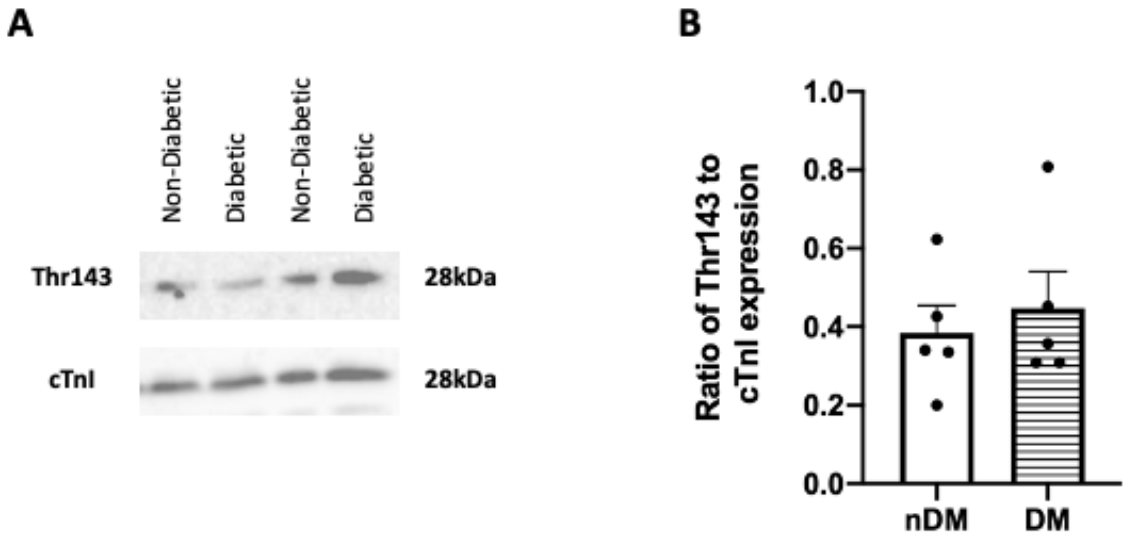

Figure 3.4. Diabetes increases phosphorylation of cTnI at threonine 143. (*A) Representative western blot images of Thr143 phosphorylated and cTnI as a control in diabetic and non-diabetic left ventricle tissue in 12-week old ZDF rats. (B) Quantitative bar graph showing the levels of phosphorylation at Thr143 in diabetic (DM) and non-diabetic (nDM) ZDF rats LV tissue at 12 weeks of age. n = 5 DM samples and 5 nDM samples. Values are represented as mean ± SEM. All these experiments were performed as duplicates.*

3.2 Calcium Sensitivity Measurements

3.2.1 Skinned Cardiomyocyte Calcium Sensitivity

The pCa_{50}, or Ca^{2+} concentration at which half-maximal tension is produced, is a measure of the Ca^{2+} sensitivity of cardiomyocytes. Figure 3.5 is an example of a tension-pCa curve for a single isolated cardiomyocyte. Due to lockdown, my project was modified to remove the training time for the Ca^{2+} sensitivity experiments. Instead, I assisted PhD candidate Angela Greenman in performing these experiments and analysed a subset of the data (discussed in this thesis). The example curve in Figure 3.5 shows data from a control non-diabetic sedentary, isolated cardiomyocyte. The results are fitted to a continuous sigmoidal curve, and the equation that fits the curve indicates the pCa_{50} of that particular cardiomyocyte. The results of each experiment with the fitted tension–pCa curve can be used to calculate the distribution of pCa_{50} within the non-diabetic sedentary (nDM SED), non-

diabetic trained (nDM EX), diabetic sedentary (DM SED) and diabetic trained (DM EX) isolated cardiomyocytes.

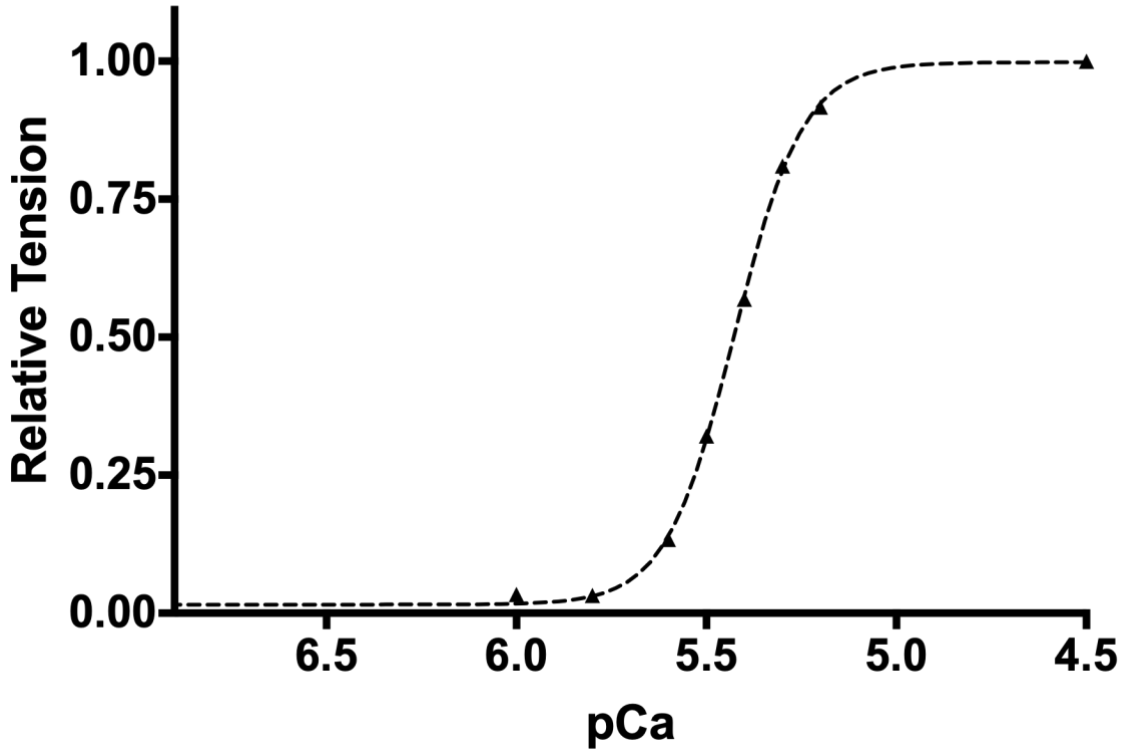

Figure 3.5. Representative tension-pCa curve. *Myofilament pCa-tension relationship for an individual sedentary nDM left ventricular cardiomyocyte (n = 1). The curve is drawn according to the parameters developed by fitting the data to the Hill equation. pCa_{50} = 5.425. Mean pCa_{50} for 20-week ZDF nDM sedentary, DM sedentary, nDM trained, and DM trained skinned cardiomyocytes can be analysed and used to find shifts indicating changes in Ca^{2+} sensitivity within tissue sample groups.*

3.2.2 Calcium Sensitivity in Response to Exercise Training

Figure 3.6 shows the mean pCa-tension curves created for diabetic trained and non-diabetic trained samples. The results indicate that the differences in pCa_{50} between the two sample groups are not significantly different. My data show a trend towards increased pCa_{50} in diabetic trained cardiomyocytes compared to non-diabetic trained cardiomyocytes (5.428 vs. 5.471, nDM EX vs. DM EX; Figure 3.6), indicated by a leftward shift. However, this data is not statistically significant, and hence no firm conclusions of altered calcium sensitivity can be supported. Again, this analysis was performed for only a subset of the animals within the

study, and when the full set of data is analysed by *Ms* Angela Greenman, we may see shifts in Ca^{2+} sensitivity indicating exercise mitigates the dysregulations in Ca^{2+} sensitivity observed in diabetic hearts.

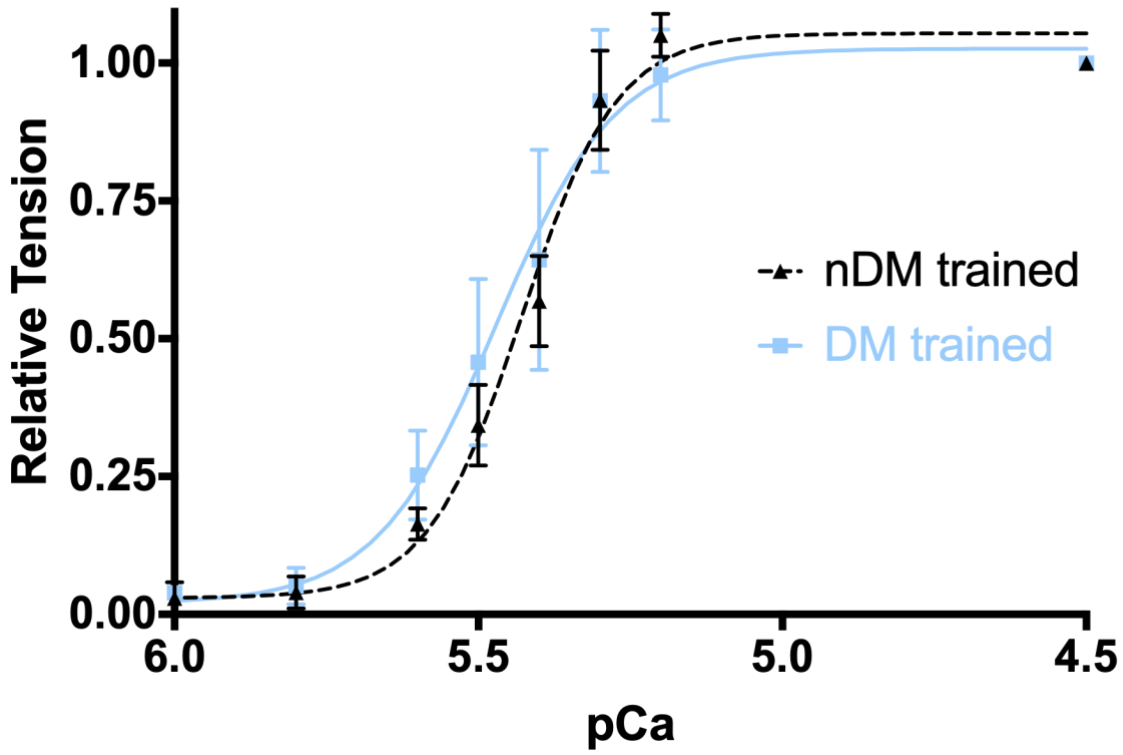

Figure 3.6. Calcium sensitivity is shifted in diabetic trained cardiomyocytes. *Myofilament pCa_{50} increased in individual skinned diabetic trained rat heart cells, indicated by a leftward shift. Mean pCa-tension relationship (where pCa = -log10[Ca^{2+}]) obtained from skinned cardiomyocytes from ZDF non-diabetic sedentary, diabetic sedentary, non-diabetic trained, and diabetic trained rats' hearts at 20 weeks of age. The pCa-tension curve is generated by normalising force at each pCa to maximal force measured in pCa 4.5. Statistics are calculated with a modified Hill equation. n = 3 diabetic trained, 3 diabetic sedentary, 3 non-diabetic trained, and 3 non-diabetic sedentary samples.*

3.3 Exercise Intervention Analysis of 20-week old nDM and DM Rats

3.3.1 Verification of Total cTnI Expression

A representative blot is presented in Figure 3.7A, showing total cTnI protein bands at 28kDa, which corresponds to the expected size. As expected, the levels of total cTnI were expressed evenly in all non-diabetic sedentary, non-diabetic trained, diabetic sedentary and diabetic trained groups. Figure 3.6B shows a quantitative assessment of total cTnI expression

in tissue from left ventricle tissue from all four sample groups of ZDF rats at 20 weeks of age. Total cTnI expression was unchanged in the left ventricle tissue across all 4 groups (0.77 ± 0.092 vs. 0.67 ± 0.036 vs. 0.69 ± 0.029 vs. 0.70 ± 0.040, nDM SED, DM SED, nDM EX, DM EX; Figure 3.7B). These results suggest that cTnI expression is the same in diabetic trained vs. diabetic sedentary vs. non-diabetic trained vs. non-diabetic sedentary ZDF rats' hearts at 20 weeks of age proceeding exercise training.

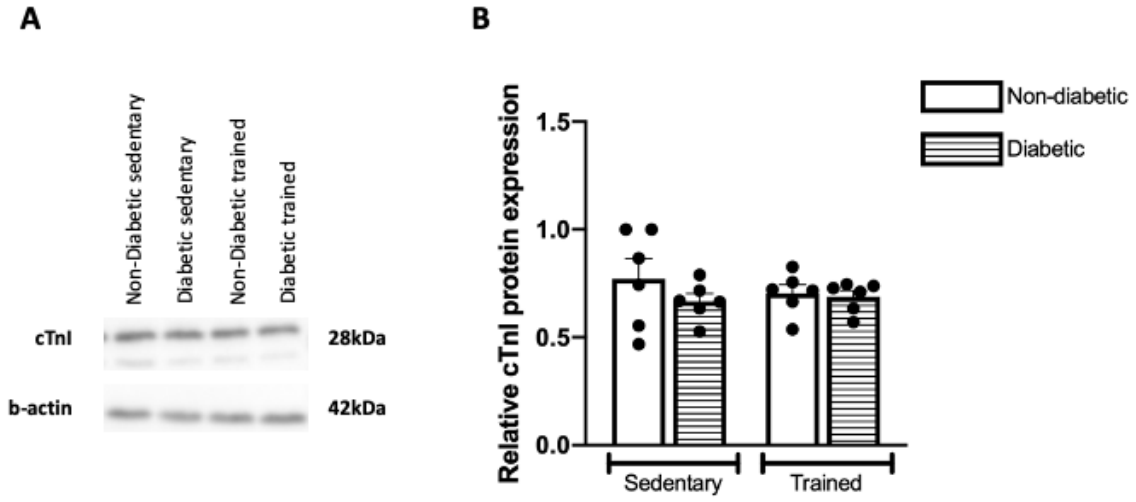

Figure 3.7. Total expression of cTnI in diabetic sedentary and trained and, non-diabetic sedentary and trained rat hearts at 20 weeks of age*. (A) Representative western blot image of cTnI and β-actin. (B) Individual value plots with a bar graph showing the quantification of cTnI in diabetic sedentary, diabetic trained, non-diabetic sedentary and, non-diabetic trained LV tissue of ZDF rats. β-actin was used as an internal control. n = 6 diabetic trained, 6 diabetic sedentary, 6 non-diabetic trained, and 6 non-diabetic sedentary samples. Values are represented as mean ± SEM. Statistics calculated with two-way ANOVA with Tukey's post hoc test. All these experiments were performed as duplicates.*

3.3.2 Serine 23/24 Phosphorylation

Expression of cTnI phosphorylation at Ser23/24 was measured in left ventricle tissue from non-diabetic sedentary, non-diabetic trained, diabetic sedentary and diabetic trained ZDF rats (Figure 3.8A). Diabetic trained rats show a significant reduction in the amount of phosphorylation at the Ser23/24 site, compared to non-diabetic trained rats (0.80 ± 0.070 vs. 0.39 ± 0.024, nDM ex vs. DM ex, $P<0.05$; Figure 3.8B). Similarly, diabetic trained rats show

a significant reduction of the amount of phosphorylation at Ser23/24 compared to diabetic sedentary rats (0.68 ± 0.058 vs. 0.39 ± 0.024, DM SED vs. DM EX, P<0.05; Figure 3.8B). These results support my hypothesis that exercise can lead to altered levels of phosphorylation at Ser23/24 in the diabetic heart.

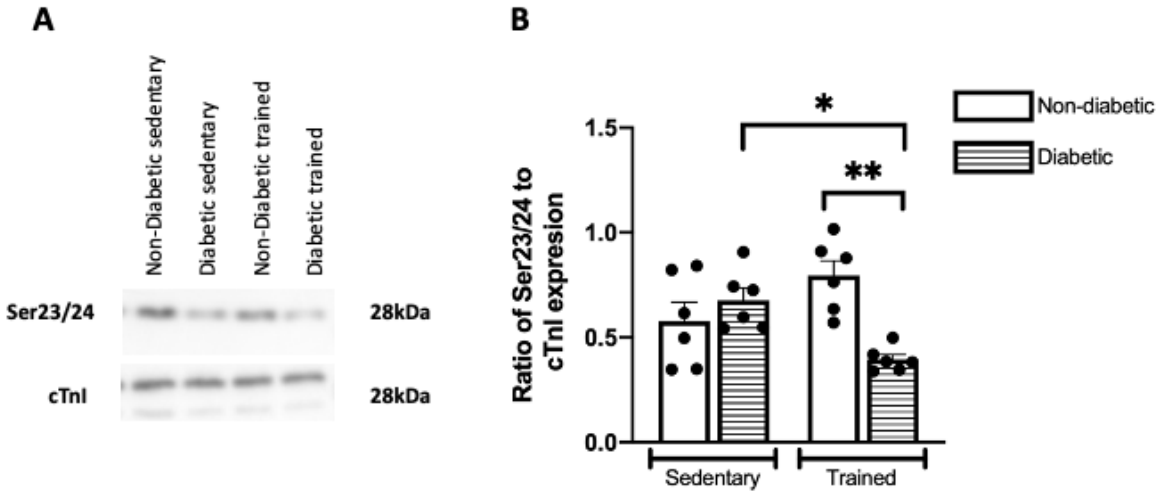

Figure 3.8. Alterations in levels of phosphorylation at Serine 23/24 in the later stages of diabetes. *(A) Representative western blot images of Ser23/24 phosphorylated and cTnI as a control in diabetic trained, diabetic sedentary, non-diabetic trained, and non-diabetic sedentary. (B) Quantitative bar graph showing the levels of phosphorylation at Ser23/24 in diabetic sedentary, diabetic trained, non-diabetic sedentary, and non-diabetic trained ZDF rats LV tissue at 20 weeks of age. n = 6 diabetic trained, 6 diabetic sedentary, 6 non-diabetic trained, and 6 non-diabetic sedentary samples. Values are represented as mean ± SEM. All these experiments were performed as duplicates. Statistics calculated with two-way ANOVA with Tukey's post hoc test. *P < 0.05 vs. diabetic sedentary. ** < 0.01 vs. non-diabetic trained.*

3.3.3 Serine 150 Phosphorylation

Expression of phosphorylation of cTnI at Ser150 was measured in left ventricle tissue from non-diabetic sedentary, non-diabetic trained, diabetic sedentary and diabetic trained ZDF rats (Figure 3.9A). No statistical significance was evident between any of the four sample groups. Diabetic trained rats show an increasing trend in the levels of phosphorylation compared to non-diabetic trained rats (0.33 ± 0.083 vs. 0.58 ± 0.075, nDM ex vs. DM ex, P = 0.14; Figure 3.9B). In addition, diabetic trained rats also show increasing trends in the levels of phosphorylation diabetic sedentary rats (0.32 ± 0.092 vs. 0.58 ± 0.075, DM sed vs. DM ex,

P = 0.18; Figure 3.9B). Overall, these results do not provide enough evidence to support my hypothesis that diabetes alters the levels of phosphorylation compared to non-diabetic cardiomyocytes.

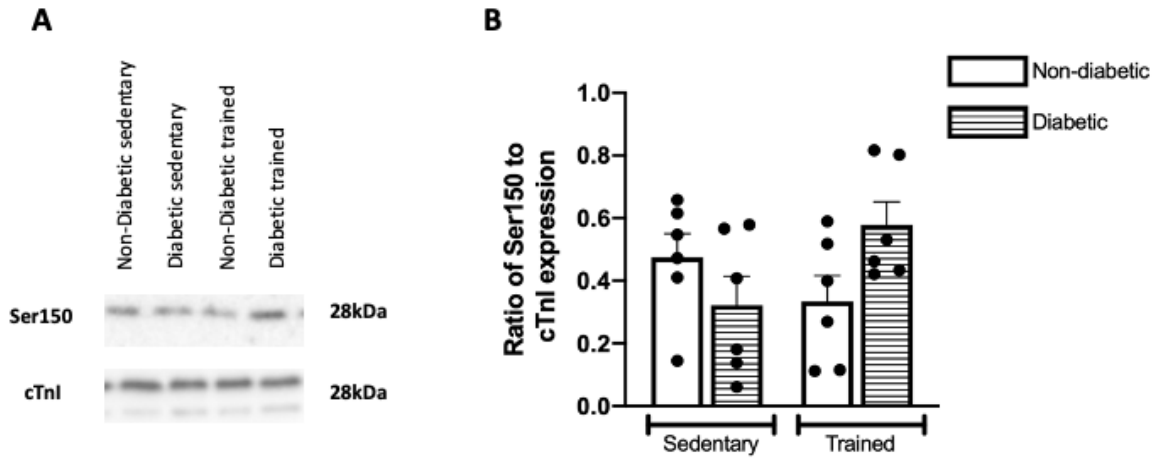

Figure 3.9. Alterations in levels of phosphorylation at Serine 150 in the later stages of diabetes. *(A) Representative western blot images of Ser150 phosphorylated and cTnI as a control in diabetic trained, diabetic sedentary, non-diabetic trained, and non-diabetic sedentary. (B) Quantitative bar graph showing the levels of phosphorylation at Ser150 in diabetic trained, diabetic sedentary, non-diabetic trained, and non-diabetic sedentary ZDF rats LV tissue at 20 weeks of age. n = 6 diabetic trained, 6 diabetic sedentary, 6 non-diabetic trained, and 6 non-diabetic sedentary samples. Values are represented as mean ± SEM. Statistics calculated with two-way ANOVA with Tukey's multiple post hoc test. All these experiments were performed as duplicates.*

3.3.4 Threonine 143 Phosphorylation

Expression of cTnI phosphorylation at Thr143 was measured in left ventricle tissue from non-diabetic sedentary, non-diabetic trained, diabetic sedentary and diabetic trained, ZDF rats (Figure 3.10A). Diabetic trained rats show a significant reduction in the amount of phosphorylation at the Thr143 site, compared to non-diabetic trained rats (1.0 ± 0.096 vs. 0.48 ± 0.070, nDM ex vs. DM ex, P<0.01; Figure 3.10B). Similarly, diabetic trained rats had a significant reduction of the amount of phosphorylation at Thr143, compared to diabetic sedentary rats (1.1 ± 0.12 vs. 0.48 ± 0.070, DM sed vs. DM ex, P<0.01; Figure 3.10B). Diabetic trained, rats also show a significant reduction in the amount of phosphorylation

when compared to the non-diabetic sedentary rats (1.1 ± 0.10 vs. 0.48 ± 0.070, nDM sed vs. DM ex, P<0.01; Figure 3.10B). These results provide enough evidence to support my hypothesis that exercise can lead to altered levels of phosphorylation at Thr143 in the diabetic heart.

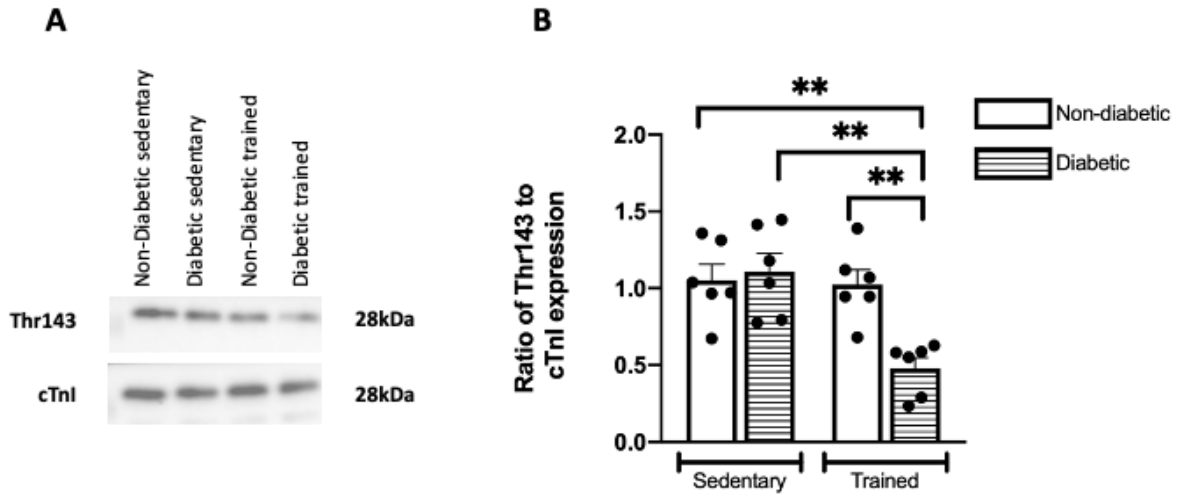

Figure 3.10. Alterations in levels of phosphorylation at Threonine 143 in the later stages of diabetes. *(A) Representative western blot images of Thr143 phosphorylated and cTnI as a control in diabetic trained, diabetic sedentary, non-diabetic trained, and non-diabetic sedentary. (B) Quantitative bar graph showing the levels of cTnI phosphorylation at Thr143 in diabetic trained, diabetic sedentary, non-diabetic trained, and non-diabetic sedentary ZDF rats LV tissue at 20 weeks of age. n = 6 diabetic trained, 6 diabetic sedentary, 6 non-diabetic trained, and 6 non-diabetic sedentary samples. Values are represented as mean ± SEM. Statistics calculated with two-way ANOVA with Tukey's post hoc test. All these experiments were performed as duplicates. **P < 0.01 vs. diabetic sedentary. **P < 0.01 vs. non-diabetic sedentary. **P < 0.01 vs. non-diabetic trained.*

4 Discussion

This project aimed to investigate the role that cTnI plays in the diabetic heart and examine whether phosphorylation at cTnI serine and threonine residue sites was altered in non-diabetic and diabetic ZDF rats. Furthermore, I investigated whether pCa_{50} is reduced in the diabetic heart initially or after eight weeks of uncontrolled hyperglycemia. Additionally, I aimed to determine whether exercise can alter phosphorylation activity and pCa_{50} responsible for cellular functional changes in the myocardium in the late stages of diabetes.

I hypothesised that in ZDF diabetic tissue at both 12 and 20 weeks of age, the results would show alterations in cTnI phosphorylation activity that would correspond to a reduction in pCa_{50}. I also hypothesised that in 20-week old ZDF rat diabetic left ventricle tissue; exercise training would cause alterations in cTnI phosphorylation activity that would correspond to an increase in pCa_{50}.

My results indicate that cTnI protein phosphorylation was altered in diabetic myocardium, suggesting a reduction in Ca^{2+} sensitivity at 12 weeks of age. Additionally, cTnI protein phosphorylation was altered significantly in trained diabetic myocardium compared to sedentary diabetic myocardium, suggesting an increase in Ca^{2+} sensitivity.

4.1 Total Cardiac Troponin I Expression Measurements

The phosphorylation of cTnI represents an important physiological mechanism for altered myofilament properties and contributes to contractile dysfunction observed in diabetic patients (Jweied *et al.*, 2005; Layland *et al.*, 2005). An initial objective of this study was to identify the total expression of cTnI and get an indication of whether it was increased,

decreased or not changed at all in diabetic cardiomyocytes compared to non-diabetic cardiomyocytes. This analysis was conducted in left ventricle tissue at 12 weeks of age as this is a well-established age to develop hyperinsulinemia and is thought to be modelling early-onset of diabetic heart dysfunction (Reed & Scribner, 1999). As expected, the protein expression of cTnI was similar between the two groups, indicating that cTnI protein expression may not change in diabetic myofilaments and non-diabetic myofilaments prior to administration of exercise. This again suggests that cTnI expression that is distinct from diabetes is not influencing the differences in levels of phosphorylation that is observed between the two sample groups.

4.2 Cardiac Troponin I Phosphorylation Measurements at 12 Weeks of Age

The type 2 diabetic heart experiences the development of cardiovascular diseases, commonly displaying symptoms of impaired diastolic performance (Baldi *et al.*, 2006). It is believed this is due to intrinsic myofilament properties being altered, affecting the cellular structure and function of cardiomyocytes (Layland *et al.*, 2005). The findings from this current study are consistent with this theory. We tested three phosphorylation sites, Ser23/24, Ser150, and Thr143, within cTnI known to modulate Ca^{2+} sensitivity (Pfitzer *et al.*, 1982; Noland *et al.*, 1989; Ouyang *et al.*, 2010; Solaro, 2011). Ser23/24 results show a significant increase in the levels of phosphorylation in diabetic rat hearts compared to non-diabetic rat hearts, indicating a functional change in the myofilament. However, the difference in the levels of phosphorylation at Thr143 were not significantly different, potentially due to variability among the animals in the protocol. In contrast, phosphorylation was significantly increased in diabetic rats' hearts at Ser150 compared to non-diabetic hearts. In line with my

hypothesis, diabetic cardiac tissue shows alterations in cTnI phosphorylation that might underpin the cellular changes in myofilament properties.

4.2.1 Modifications at Ser23/24

In Figure 3.2, diabetic ZDF tissue shows a significant increase in the amount of phosphorylation at Ser23/24 compared to non-diabetic ZDF tissue. Based on prior studies, this result suggests a reduction in Ca^{2+} sensitivity that may be linked to the upregulation of PKA activity (Figure 4.1). When considering PKA phosphorylation of cTnI, literature classifies the reduction in myofilament Ca^{2+} sensitivity as a result of the phosphorylation at serine residues in positions 23 and 24 of the N-terminal (Solaro & Rarick, 1998; Noguchi *et al.*, 2004; Solaro, 2011). Myofilament Ca^{2+} desensitisation and the rate of Ca^{2+} dissociating from TnC is characteristic of PKA-dependent phosphorylation, which is mediated by β-adrenergic stimulation (Layland *et al.*, 2005), resulting in acceleration of relaxation (Hoh *et al.*, 1988). Nonetheless, multiple studies take contrary positions showing β-adrenergic stimulation results in no change in the rate of cross-bridge cycling or shortening velocity, in addition to Ca^{2+} sensitivity (Hofmann & Lange 3rd, 1994; Janssen & De Tombe, 1997; Johns *et al.*, 1997). Although we do not directly measure PKA or β-AR expression, this data would support others' conclusions that β-AR signalling is impaired or desensitised in the diabetic heart (Thaung *et al.*, 2015).

Another possible explanation based on the current literature is that the observed alterations in phosphorylation are a consequence of PKG-dependent phosphorylation. Evidence generated supports the concept that PKG is also contributing to the phosphorylation of cTnI at Ser23/24 with comparable properties to those of PKA (figure 4.1), albeit, at slower rates than PKA (Blumenthal *et al.*, 1978). Crucially, PKG I expression is identified in

cardiomyocytes (Wollert *et al.*, 2003) and the treatment of skinned cardiac preparations with PKG reduced myofilament Ca^{2+} sensitivity (Pfitzer *et al.*, 1982). Conversely, there is still limited evidence on the responses of PKG on cTnI phosphorylation and Ca^{2+} sensitivity in the myofilament. The difficulty in pinpointing the precise effects of PKG on cTnI phosphorylation is due to the uncertainty surrounding the impact of PKG on other proteins, including the reduction of L-type Ca^{2+} current or increased sarcoplasmic reticulum Ca^{2+} uptake (Shah, 1996).

It is possible, therefore, that the results I observed are a consequence of PKA and PKG upregulation and that these results might support the idea that impaired β-AR responsiveness contributes to an increase in Ser23/24 phosphorylation. However, further research is required, as the modifications in diabetic myofilaments might be unrelated to Ser23/24 phosphorylation or the observed Ser23/24 phosphorylation events may be connected to various other cardiac kinases.

4.2.2 Modifications at Thr143

In Figure 3.4, diabetic ZDF tissue did not show amounts of phosphorylation that were significantly different from that observed in non-diabetic ZDF tissue. The functional effects of Thr143 phosphorylation in the myofilament are not as well understood. Prior studies have noted that cTnI can be phosphorylated by PKC at Thr143, resulting in desensitisation of the myofilaments (Wang *et al.*, 2006) (Figure 4.1). This could be due to the strong relationship between diabetes and PKC reported in the literature (Jweied *et al.*, 2005). PKC activation has shown both negative (Takeishi *et al.*, 1998; Montgomery *et al.*, 2002) and positive inotropic effects (Pi *et al.*, 2002), as well as negative (Takeishi *et al.*, 1998) and positive lusitropic

effects (Westfall & Borton, 2003), suggesting numerous myofilament-based and non-myofilament effects of PKC.

Another possible explanation for this, is the functional consequence of phosphorylated Thr143 might be dependent on communicating with Ser43/45 (Lang *et al.*, 2017), as the literature suggests functional effects associated with both amino acid residue sites are attenuated or strengthen depending on the secondary phosphorylation. It has been demonstrated that secondary phosphorylation at PKC sites Thr143 and Ser43/45 could help to maintain homeostatic contractile function during chronic cTnI phosphorylation as observed in diabetic hearts. Lang et al. (2017) found Thr143 phosphorylation heightened the effects of Ser43/45 phosphorylation, resulting in a reduction in Ca^{2+} sensitivity. It can thus be suggested that multiple phosphorylation sites may interact together and change cellular functions.

4.2.3 Modifications at Ser150

In Figure 3.3, the results show that diabetic ZDF tissue had decreased phosphorylation at Ser150 when compared to non-diabetic ZDF tissue. Prior literature indicates that a decrease in AMPK activity leads to a reduction in cTnI phosphorylation at Ser150 (Figure 4.1). AMPK is a serine/threonine-protein kinase, and evidence signifies that AMPK can phosphorylate cTnI (Hardie & Carling, 1997; Kobayashi & Solaro, 2005; Oliveira *et al.*, 2007; Sancho Solis *et al.*, 2011). Given this, the evidence established cTnI Ser150 phosphorylation as functionally significant, indicating that Ca^{2+} regulation was altered though cTnI pseudo-phosphorylation, affecting the interaction between cTnI and cTnC (Ouyang *et al.*, 2010). Ouyang et al.'s (2010) study focused on the selectivity of AMPK for cTnI phosphorylation sites and confirmed that Ser150 and Ser23 were targeted quite selectively as Ser22 and Thr143 were not observed to be phosphorylated. The current study results indicate that the

reduction in Ser150 phosphorylation could be due to a reduction in AMPK activity resulting in a decrease in Ca^{2+} sensitivity. Moreover, this current study was successful in identifying that diabetes might play a role in the dysregulation of Ser150 phosphorylation.

Collectively, these findings from Figure 3.2, 3.3, and 3.4 could explain why alterations at specific cTnI phosphorylation residue sites affect cardiac myofilament properties, that may underlie the decreased contractile function of the ventricle often characterised in diabetic cardiomyopathy. This is based off similar findings indicating that diabetes can result in altered myocardium functionality resulting in weaker force generation, characteristic of contractile dysfunction (Diffee *et al.*, 2001). These results provide evidence of alterations in cTnI phosphorylation in diabetic tissue.

4.3 Single-cell Calcium Sensitivity Measurements

There is limited research on the relationship between myofilament Ca^{2+} sensitivity and diabetic cardiomyocytes, but collectively they demonstrate that Ca^{2+} sensitivity is reduced in human diabetic cardiomyocytes (Jweied *et al.*, 2005). Based on these findings, we hypothesised that cardiomyocytes from diabetic rat hearts would show decreased pCa_{50} values compared to non-diabetic counterparts. Interestingly, diabetic trained cardiomyocytes showed a trend towards increased pCa_{50} (though this was not significant), potentially indicating an increase in Ca^{2+} sensitivity compared to non-diabetic trained cardiomyocytes (Figure 3.6). If this difference became significant once the entire set of animals is analysed, it would stand in contrast to published findings and our hypothesis. This discrepancy could be attributed to a compensatory mechanism to maintain normal systolic function that could also have adverse effects on the diastolic function as the disease progresses. Interestingly, the data indicates an increase in pCa_{50} values in skinned cardiomyocytes from diabetic trained rats compared to

diabetic sedentary rats, indicating sensitised diabetic myofilament proteins, this would be evidenced by a leftward shift in the tension-pCa relationship. This means more tension is developed at submaximal Ca^{2+} concentration.

These results corroborate findings which suggested that the reduction in Ca^{2+} sensitivity in these works provide an explanation as to why reductions in contractility or compensation to improve rates of relaxation is observed in the diabetic heart (Piccini *et al.*, 2004). This result may be explained by the fact that exercise induces increases in isometric tension observed in myocardial preparations (Tibbits *et al.*, 1981) and increases in cell shortening observed in single cardiomyocytes (Moore *et al.*, 1993). Previous work examining the cellular basis underlying the increase in myocardial contractility induced by exercise has focused on training-induced alterations in mechanisms that may increase intracellular Ca^{2+} concentration during activity, however many have shown inconclusive results (Fitzsimons *et al.*, 1990; Laughlin *et al.*, 1991; Lankford *et al.*, 1998). Although the present study is one of the first to directly measure Ca^{2+} sensitivity of tension in relation to exercise training in diabetic hearts, with a greater sample size, our data might suggest that Ca^{2+} sensitivity may be increased in trained diabetic cardiomyocytes.

4.4 Cardiac Troponin I Phosphorylation Measurements at 20 Weeks of Age

It is well-established that exercise as an intervention for CVD results in positive adaptations of cardiac function, thus improving the health of type 2 diabetic patients. Whilst the role of chronic exercise training in patients with a cardiovascular disease has been previously investigated (Diffee *et al.*, 2001; Diffee & Nagle, 2003; Lear *et al.*, 2017), its role in the myofilaments of a diabetic heart is relatively unknown. Data from this study may

provide a possible explanation as to why the pathological effects of diabetes can be improved by exercise. The data shows that the diabetic left ventricle tissue could improve the Ca^{2+} sensitivity of the myofilament following moderate-intensity exercise training, as they exhibit alterations in phosphorylation levels contrary to non-diabetic counterparts.

4.4.1 Sedentary Response

We tested the same three residue sites, investigated in 12-week old rats' hearts, specific to cTnI, Ser23/24, Ser150, and Thr143, within cTnI known to modulate Ca^{2+} sensitivity (Pfitzer *et al.*, 1982; Noland *et al.*, 1989; Ouyang *et al.*, 2010; Solaro, 2011). A note of caution is due here since the differences observed were not significant; therefore, a conclusion cannot be made, however, phosphorylation at Ser23/24 was trending towards an increase in the diabetic sedentary hearts compared to the non-diabetic hearts. This also accords with our earlier observations along with literature, which indicates that diabetes could be intervening in the pathways regulating phosphorylation activity by PKA and PKC at specific residue sites within cTnI. Consequently, this results in changes to protein structure and function, which might impair Ca^{2+} sensitivity. Phosphorylation at Ser150 was also trending towards a decrease in the diabetic sedentary heart tissue compared to non-diabetic hearts. This finding was also reported in my 12-week data suggesting a change in the levels of phosphorylation, possibly due to dysregulations in the AMPK pathway.

4.4.2 Ser23/24 Phosphorylation in Response to Exercise

The second aim of this study sought to determine if exercise could intervene in the amount of phosphorylation observed in diabetic rats' heart and reverse the pathological effects that are observed in a diabetic heart. One of the most interesting findings was that observed in Figure 3.7 showing that phosphorylation at Ser23/24 in diabetic trained rats'

hearts was decreased when compared to non-diabetic trained, and diabetic sedentary rats' hearts. This implies that, upon moderate-intensity exercise training, the amount of phosphorylation is altered in the diabetic cardiomyocytes.

Very little research has investigated the role of exercise as a mechanism for altering the amount of cTnI phosphorylation by which mechanical properties are regulated in the diabetic heart. Interestingly, the results showing a reduction in the amount of phosphorylation observed at Ser23/24 suggests an increase in Ca^{2+} sensitivity which may be explained by the relationship between PKA, β-AR protein expression and exercise (Haley *et al.*, 2015). Research by Leosco et al. (2013) has illustrated that exercise reverses β-AR dysfunction through modulating G-protein-dependent adenylyl cyclase activation and restoring cardiac receptor levels and activity. This becomes important because several reports have stated that diabetes commonly results in β-AR dysfunction whereby PKA activation is increased (Jweied *et al.*, 2005; Leosco *et al.*, 2013; Haley *et al.*, 2015). However, exercise might provide a way to improve β-AR signalling and responsiveness in type 2 diabetic hearts. Similar observations also have been reported in post-ischemic hypertrophied failing myocardium, demonstrating that exercise evokes similar effects on β-AR signalling and responsiveness, resulting in the enhanced cardiac inotropic state (Leosco *et al.*, 2013). Furthermore, β-AR responses were improved in intact cardiomyocytes of diseased hearts illustrating that moderate-intensity exercise training might restore β-AR signalling alterations in the myocardium that contributes to left ventricle dysfunction. Therefore, exercise training could be a significant factor causing a shift in the phosphorylation activity at Ser23/24.

4.4.3 Thr143 Phosphorylation in Response to Exercise

As previously mentioned, the functional effects of phospho-Thr143 are still relatively unknown, although it is believed to be a PKC target site, along with Ser23/24. Interestingly, Figure 3.9 shows that the amount of phosphorylation observed at Thr143 was significantly altered from diabetic trained rats hearts to diabetic sedentary hearts, indicating that exercise might be altering PKC-dependent phosphorylation in the diabetic heart. It is well-established that contractility is regulated by cTnI phosphorylation by PKC and that PKC is activated in diabetes (Kirk *et al.*, 2009; Lang *et al.*, 2015). However, the activation of PKC-dependent phosphorylation at Thr143 is associated with a reduction in Ca^{2+} responsiveness, in agreement with impaired contractility characteristic of the type 2 diabetic heart.

In the case of the trained diabetic rats, exercise might be partially inhibiting the PKC pathway targeting Thr143, returning the pathway to a homeostatic activity level. Another alternative explanation is that exercise is heightening the cross-talk between Thr143 and Ser43/45, as previously mentioned in section 4.2.2. Collectively, these might be contributing to reduced phosphorylation, allowing the myofilaments to increase their Ca^{2+} responsiveness, inducing the heart to function at near-maximum kinetics. Previous work using reconstituted skinned fibre bundles in which endogenous cTnI was mutated to mimic constitutive phosphorylation showed that phosphorylation at Thr144 caused a reduction in Ca^{2+} sensitivity and filament sliding velocity (Burkart *et al.*, 2003). However, PKC activation is known to occur through several other pathways also associated with diabetes (Jweied *et al.*, 2005). Prior studies have noted that serum levels and hyperglycemia, commonly observed in diabetic patients, may contribute to the upregulation, eliciting effects that also impair the cardiac tissue (Shiba *et al.*, 1993; Karar *et al.*, 2015). Overall, it is encouraging that the results from trained diabetic rats' hearts show reductions in amounts of phosphorylation, which suggest an

increase in Ca^{2+} sensitivity, and this could ultimately lead to an improvement in cardiac contractility of the diabetic heart. To develop a full picture of cTnI phosphorylation at Thr143 and the implications exercise is causing, might require further research, in particular, to fully understand the importance of potential cross-talking between Ser43/45 and Thr143.

4.4.4 Ser150 Phosphorylation in Response to Exercise

One unanticipated finding was that moderate-intensity exercise had little effect on the levels of phosphorylation at phospho-Ser150. Figure 3.8 illustrates alterations in the levels of phosphorylation at Ser150 between the diabetic trained cardiomyocytes, diabetic sedentary, and non-diabetic trained cardiomyocytes; however, these differences are only indicating potential trends. The trained diabetic samples demonstrate a reducing trend in the levels of phosphorylation, which suggest an increase in Ca^{2+} sensitivity. Literature has indicated that the phosphorylation at Ser150 might be associated with the interaction of cTnI with cTnC affecting thin filament Ca^{2+} modulation, but also the possibility of cross-talking with PKA-dependent Ser23/24 phosphorylation (Nixon *et al.*, 2012). The role of AMPK to modulate energy substrate availability as a regulator of heart function has been established (Nixon *et al.*, 2012); however, the literature also suggests that AMPK is directly coupled to a mechanical effect of contraction at the myofilament level following Ser150 phosphorylation (Arad *et al.*, 2007). Although our results were not significantly different, they show trends that encourage this idea. Exercise training stimulated an increasing trend in the levels of phosphorylation, in line with literature which states that alterations in the AMP/ATP ratio can result in AMPK phosphorylation. This often occurs in a diseased heart due to the increased stress on the heart, increasing Ser150 phosphorylation (Sancho Solis *et al.*, 2011). Exercise might provide a mechanism to balance the AMP/ATP ratio as such coupling of AMPK increasing energy substrate availability to a cTnI Ser150-enhanced myofilament response to

Ca^{2+}, might be beneficial to maintaining enhanced contractility during times of stress without the enhanced energetic cost of increased Ca^{2+} cycling.

The effects of AMPK-dependent phosphorylation at Ser150 might also contribute to blunt cTnI PKA-dependent phosphorylation, which literature suggests results in an increase in Ca^{2+} sensitivity (Hoh *et al.*, 1988). This current study indicates that exercise alters phosphorylation at Ser23/24, suggesting an increase in Ca^{2+} sensitivity which, as previously discussed, could be due to a return to full functionality of β-AR stimulation. However, this could also be due to cross-talking within cTnI to blunt myofilament PKA-dependent functional effects, resulting in uncoupling of the myofilament to the β-AR response. Nevertheless, whether cTnI phosphorylation at the AMPK site is indeed elevated in the diabetic trained heart cannot be established, since we did not have sufficient results to make this conclusion.

Although the mechanisms that underlie cardiac myofilament dysfunction in association with diabetes and the amount of phosphorylation occurring cannot be determined from this study, it is possible that altered myofilament contractile protein phosphorylation by PKA, PKC, PKG, and AMPK at Ser23/24, Ser150 and Thr143, plays a significant role in this process.

Regardless of the mechanism, levels of cTnI phosphorylation and changes observed in the diabetic rats' hearts might be affected by moderate-intensity exercise, altering the Ca^{2+} sensitivity of the myofilaments through changes in levels of phosphorylation, thus, affecting the global function of the heart. An increase in Ca^{2+} sensitivity would provide one explanation for the improvement in contractility due to less Ca^{2+} required to generate a normal amount of

force in the diabetic cardiomyocyte. Moreover, it could be an explanation for diastolic dysfunction and arrhythmias commonly observed in diabetic patients. Viewed another way, the relatively preserved global systolic function observed in diabetic patients, in general, could be due to a reduction in Ca^{2+} sensitivity compensated for by a potential maladaptive increase in Ca^{2+} to generate a force similar to a non-diabetic cardiomyocyte.

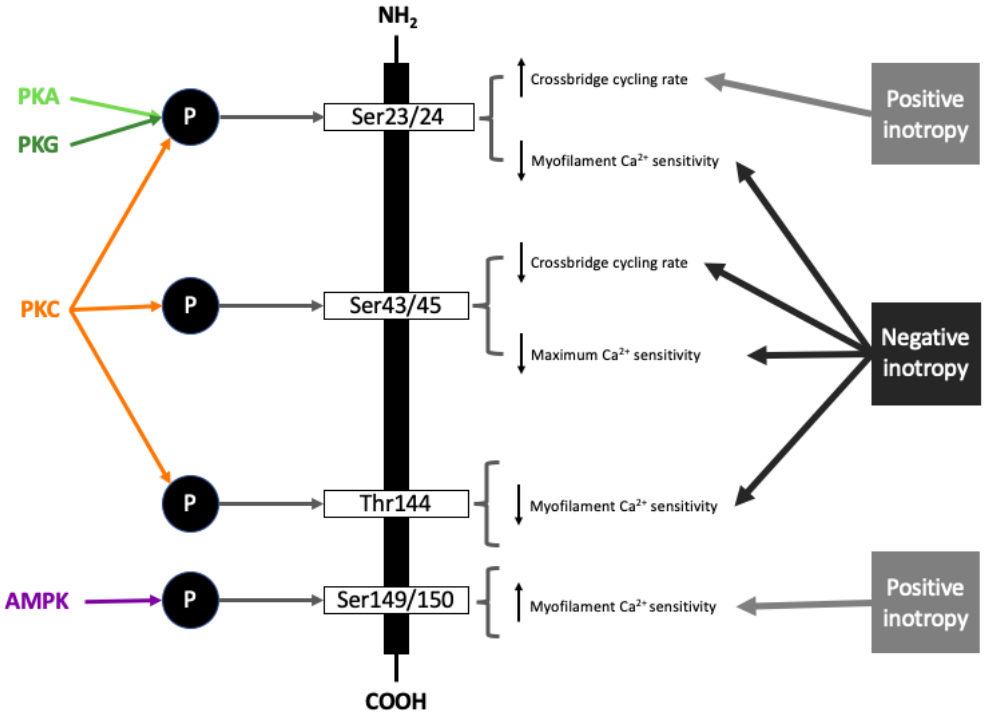

Figure 4.1. **Schematic diagram showing the major cTnI phosphorylation pathways involved in the regulation of cardiac function.** The specific sites being phosphorylated in combination with the activity of protein phosphatases regulates the changes in contractility of cTnI phosphorylation. Phosphorylation is represented by P and the kinases phosphorylating the residue sites are located left of the arrow. The changes in myofilament properties that result in alterations of heart function are indicated on the right. Amino acid and carboxy termini of cTnI are indicated by NH_2 and COOH, respectively. (Gold, 2020, Original figure).

This study provides the first comprehensive assessment of the effects of exercise on myofilament protein, cTnI, phosphorylation in a diabetic rats' heart. The findings from the present study, while preliminary, suggest that a moderate-intensity exercise scheme may modulate myofilament proteins and reverse phosphorylation activity that may correspond to an increase in Ca^{2+} sensitivity. However, further investigation is required to expand our

knowledge of the troponin complex, and the possibility that exercise is causing alterations in phosphorylation activity that enhance cardiac structure and function in a diseased heart.

4.5 Limitations

Several limitations must be taken into account with the findings from this honours project. Firstly, there was concern surrounding the stability of the post-translational modifications in the 12-week old tissue, as they are known to be less stable over time. Literature suggests that, in cTnI, by the seventh day of storage, there is a significant reduction in phosphorylation taking into consideration anaesthetic regimen and sample storage conditions (Utter *et al.*, 2015). Additionally, there was an overall decrease in myofilament phosphorylation from samples stored from 30 to 90 days. This study attempted to complete experiments as quickly as possible; however, it is possible that we were unable to measure phosphorylation in its physiological state. Precautions, such as storage on ice and protease inhibitors, were used to prevent changes in post-translational modifications during experimentation.

Secondly, the length of the exercise protocol was also subject to technical difficulties. Previous studies have used an intervention period of 10-weeks; however, our intervention period was only 8-weeks long, not including the familiarity period which made our protocol two weeks shorter than other protocols (using healthy rats). An 8-week period was chosen due to a significant decline in quality of life for the ZDF rats, which develop cataracts and malaise around 20 weeks of age (Katsuda *et al.*, 2014). We decided it was unethical to train the rat past this point. Another factor limiting the ability to extend the running protocol is that ZDF diabetic rats are not considered to develop diabetes until 12 weeks of age (VanHoose *et al.*, 2010).

4.6 Clinical Implications

The current data highlights the important role performed by cTnI phosphorylation at Ser23/24, Ser150, and by Thr144 in the alteration of myofilament function in the diabetic heart. The profound effects of cTnI phosphorylation are determined by the combination of kinases and phosphatases activated and the specific sites phosphorylated. Primarily, the Ca^{2+} sensitivity of the myofilament may be dramatically affected by differential phosphorylation at numerous positions within cTnI. Phosphorylation of cTnI at Ser23/24 reduces Ca^{2+} sensitivity, while phosphorylation at Thr143 reduces contractile force; effectively impairing the function of the heart cell. In contrast, phosphorylation at Ser150 increases Ca^{2+} sensitivity and contractility, improving function. Under physiological conditions, these effects on contractile function impact the fine-tuning in cardiac function performance, thus optimising cardiac performance under conditions of different states. These findings may help us to understand what is occurring under pathological conditions of diabetes and how it results in disruptions to cellular homeostasis, which could result in impaired contractile function commonly observed in diabetic patients.

The present study also raises the possibility that exercise, at least in part, is modulating the mechanisms responsible for altering the amount of phosphorylation of various amino acid residues within cTnI, a process by which mechanical properties are changing in the diabetic heart. The principal theoretical implication of this study is that the alterations occurring to the levels of phosphorylation suggest an increase in Ca^{2+} sensitivity which, functionally, would result in improvements in cardiac contractility and could ultimately lead to an improvement in the patient's quality of life. With further research, a drug could be developed that targets the same mechanism that exercise is targeting. Studies to develop a clinically suitable drug that

mimics the same effects as the exercise protocol should be considered to minimise the substantial impact that heart disease has on patients suffering from type 2 diabetes.

4.7 Future Directions

In this thesis, despite these promising results, questions remain. Further research should be undertaken to investigate whether high-intensity exercise training can elicit greater cardiac adaptations than moderate-intensity exercise, as this current training protocol was modelled on a low-intensity running intervention previously used in ZDF rats (VanHoose *et al.*, 2010). The speed and duration of the running were increased from the previous study to better match rat-running protocols shown to effectively increase aerobic capacity after ~10 weeks of training which closely model a moderate-intensity training protocol (Diffee *et al.*, 2001; Wisloff *et al.*, 2007). However, it would be of interest to investigate high-intensity exercise as the literature indicates that vigorous exercise in cardiac patients is a safe intervention that improved VO_2max, cardiac output, and left ventricular stroke volume (Ekblom *et al.*, 1968). Similarly, data quantifying the safety of high-intensity interval training in diabetic patients is limited, with only one study dealing with the risk of moderate versus high-intensity exercise in cardiac patients (Rognmo *et al.*, 2012). Interestingly, their results suggest that cardiac complications were 1 per 129,466 moderate-intensity training hours versus 1 per 23,182 high-intensity training hours. More recently, studies have shown that high-intensity interval training, integrating short burst of high-intensity exercise separated by moderate-intensity intervals, heightened left ventricular contractility and reversed remodelling of the left ventricle (Wisloff *et al.*, 2007).

Another area that could benefit from further research is the pCa_{50} of isolated diabetic cardiomyocytes in response to training, as the project under discussion obtained limited data

on the Ca^{2+} response to exercise training in diabetic rats' hearts. Future studies may wish to examine whether the trends observed in the ZDF trained rats' isolated cardiomyocytes are observed in human trained diabetic cardiomyocytes. Recent unpublished research from our group has indicated that in human diabetic cardiomyocytes, there is a decrease in pCa_{50} when compared to non-diabetic cardiomyocytes. This reduction in pCa_{50} could be due to changes in myofilament structure and functions, in particular cTnI. Although their study did not test the effect of exercise, it is believed that exercise can reverse this pathological mechanism of diabetes to increase the Ca^{2+} sensitivity (Moore *et al.*, 1993); however, research has not fully identified the pCa_{50} in trained diabetic cardiomyocytes. Future projects may test both human and rat heart tissue, following a high-intensity exercise protocol. As we cannot pre-determine human type 2 diabetic patients, this observation could be done following a training protocol for some time after their diagnosis.

Further studies could be continued using the animal model of type 2 diabetes - the ZDF rat. With evidence from the present study in addition to work done by Diffee et al. (2001), associating alterations in cTnI phosphorylation through exercise training with an increase in Ca^{2+} sensitivity, the effects this has on the contractile mechanisms in the diabetic heart following a training protocol remains to be elucidated. An increase in pCa_{50} would result in a greater level of isometric tension generation at the same intracellular Ca^{2+} concentration; this has been widely reported with effects of training on isomeric tension generation in the myocardium (Diffee *et al.*, 2001). It has been suggested that a training-induced increase in Ca^{2+} sensitivity of the myofilaments would be consistent with literature demonstrating that in trained cardiomyocytes, the contractile response is more sensitive to intracellular and extracellular Ca^{2+}. Therefore, future studies may wish to examine whether exercise training

would lead to an increase in Ca^{2+} sensitivity resulting in an increase in contractile force in the myocardium through echocardiography and Langendorff apparatus.

To develop a full picture, another research question that could be asked includes investigating the kinases responsible for the phosphorylation at the various amino acid residues within cTnI. The decrease in pCa_{50} in diabetic hearts is associated with high levels of cTnI phosphorylation, including the PKA and PKG sites Ser23/24 (Messer *et al.*, 2007), moreover, increased PKC activity at Thr144 and Ser43/45 is also reportedly increasing pCa_{50} in diabetic hearts (Layland *et al.*, 2005). Equally important, decreased AMPK activity might be causing a decrease in phosphorylation at Ser150. Such an effect could be studied in left ventricle tissue of a ZDF rat via enzyme activity assays for protein kinases – PKA, PKC, PKG, and AMPK.

4.8 Conclusion

The purpose of this honours project was to determine the role of cTnI in diabetic hearts and investigate whether exercise intervened in this mechanism. These findings suggest that altered phosphorylation of cTnI residues led to changes in cellular function in the diabetic heart and, further, that these alterations could be mitigated via exercise training. With the data gathered in this project, I was able to accept my hypotheses. The experiments show a potential mechanism to reverse the pathological effects of type 2 diabetes in the cardiovascular system, lending support to the notion that, currently, exercise is the best therapeutic option for type 2 diabetic patients. I have outlined various future directions of research, which may aid in understanding the impact of diabetes with the interaction of exercise in modulating myofilament protein properties.

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